Allosteric effects of taxol and kinesin on single isoform microtubule lattices

by

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Declarations

This thesis is my own work for submission to the University of Warwick for the degree of Doctor of Philosophy. It has neither been published nor submitted in any application for a degree at another university.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ADP</td>
<td>Adenosine 5’-diphosphate</td>
</tr>
<tr>
<td>AMPPNP</td>
<td>Adenosine-5’-[(β,γ)-imido]triphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>EB protein</td>
<td>End binding protein</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscope</td>
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<tr>
<td>GDP</td>
<td>Guanosine 5’-diphosphate</td>
</tr>
<tr>
<td>GMPCPP</td>
<td>Guanosine-5’-[(α, β)-methylene]triphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>Guanosine-5’-(γ-thio)-triphosphate</td>
</tr>
<tr>
<td>γ-TuRC</td>
<td>γ-tubulin ring complex</td>
</tr>
<tr>
<td>MAPs</td>
<td>Microtubule-associated proteins</td>
</tr>
<tr>
<td>MDA</td>
<td>Microtubule-destabilising agent</td>
</tr>
<tr>
<td>MSA</td>
<td>Microtubule-stabilising agent</td>
</tr>
<tr>
<td>MTA</td>
<td>Microtubule-targeting agent</td>
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<tr>
<td>PTMs</td>
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Abstract

In classical microtubule dynamic instability, all the important reactions occur at the microtubule tips. In addition to these, recent work has revealed dynamic, functionally important changes in the core microtubule lattice, including conformational changes driven by allosteric effectors. My research questions are: How does dynamic instability change for different metazoan tubulin isoforms? Do different isoforms have different lattice mechanics? Do different isoforms respond differently to drugs and kinesin?

I show that single isoform $\alpha_{1b}/\beta_3$ and $\alpha_{1c}/\beta_{4b}$ microtubules depolymerise at markedly different rates and both of which respond differently to taxol. When present during microtubule assembly, 500 nM taxol produces localised kinks in $\alpha_{1c}/\beta_{4b}$ microtubules but not $\alpha_{1b}/\beta_3$ microtubules, which I assign to local lattice expansion. Taxol washout relaxes the kinks. Further, my different single isoform microtubules in kinesin motility assays differs remarkably where GDP-taxol $\alpha_{1b}/\beta_3$ microtubules slide appreciably slower than GDP-taxol $\alpha_{1c}/\beta_{4b}$ microtubules. In microtubules with a leading $\alpha_{1b}/\beta_3$ segment and a faster moving trailing $\alpha_{1c}/\beta_{4b}$ or pig brain microtubule segment, the trailing segments are impeded and forced into a series of tight loops.

My data show that despite modest differences in their primary sequences, different metazoan tubulin isoforms have markedly different mechanical and dynamic responses to taxol, kinesin, and nucleotides. I discuss the range of this allosteric communication, and the possible implications of my findings for the chemical biological applications of taxol in medicine and elsewhere.
Chapter 1

Introduction

1.1 Microtubules are dynamic hollow cylinders built from a ring of protofilaments

Microtubules exist in almost all eukaryotic cells and play indispensable roles in their lives. Together with other cytoskeletal components, microtubules support and maintain cell shape. In addition to their structural role, microtubules also participate in cell division and intracellular transport. As a major provider of structural stiffness to almost all eukaryotic cells, microtubules are hollow cylinders with typically a 25 nm outer diameter. Microtubules in vivo usually consist of 13 protofilaments, each of which is built of tubulin heterodimers joined head-to-tail. These dynamic structures grow and shrink by addition and dissociation of tubulin heterodimers, which are composed of highly similar \( \alpha \) and \( \beta \) tubulin monomers that are only stable as dimers. Some organisms express several \( \alpha \) and \( \beta \) tubulins which are highly similar in amino acid sequences to form microtubules, further discussed in Section 1.3.

Microtubules, along with microtubule-associated proteins (MAPs), attach to sister chromatids to form mitotic spindle to allow metaphase plate for-
mation, followed by chromosome segregation. Due to their participation in cell division, microtubules have also become a target for cancer chemotherapy. However, the emergence of microtubule-targeting agent (MTA) resistance in patients has been associated with altered tubulin composition. Thus, there is an urgent need to study how these drugs interact with individual tubulin component.

Microtubules are also essential for efficient intracellular transport where they serve as train track to allow bidirectional transport. Cargo-bearing molecular motors dynein and kinesin perform respective retrograde and anterograde transport of intracellular materials such as neurotransmitter and mitochondria. This directional transport is especially crucial in neurons as transport of large molecules over a long distance by diffusion is often inefficient.

Due to the central importance of microtubules, tubulin is highly conserved throughout the eukaryotic tree of life. The last eukaryotic common ancestor (LECA) is thought to have contained 13-protofilament microtubules, which have therefore been conserved for over $10^9$ years of evolution across protists, fungi, plants and animals [1].

Although microtubules carry out “common functions” across different eukaryotes, namely cell division and intracellular transport, more complex organisms tend to express more different tubulins. For example, 8 $\alpha$ and 10 $\beta$ tubulins have been found in human whereas only 2 $\alpha$ and 1 $\beta$ tubulins have been identified in yeast, suggesting different tubulins may specify different functions, further discussed in Section 1.3. However, both $\alpha$ and $\beta$ tubulins are highly self-similar in amino acid sequences, raising a question about the exact distinctions among these tubulins. In addition, $\alpha$ and $\beta$ tubulins also share similar 3D structures. It is only until recent years that researchers are able to distinguish $\alpha$ and $\beta$ tubulins from cryo-EM structures of microtubules without fiducial markers due to their almost identical overall 3D conformations. Although having similar structures, $\alpha$ and $\beta$ tubulins have distinct functions and provide different binding recognition sites for interacting partners.

Despite the advances in cryo-EM instrument and image analysis al-
gorithms, we are not yet able to identify individual tubulin isoforms from microtubules assembled from a mixture of different tubulin isoforms. Recently, increasingly emerging data revealed the conformational and dynamic differences in different single isoform microtubules after recombinant single isoform tubulin purification using baculovirus expression system has become available, see Section 1.9.1 [2–4]. Combined structural studies and the fact that some tubulin isoforms have tissue specific expression, particular tubulin isoforms may play unique roles in cells. In addition, mutation and overexpression of certain tubulin isoforms have been associated with cancer and other diseases which call for the need to understand the implications of different tubulin isoforms.

Microtubules in mammals are predominantly made up of 13 protofilaments [5]. However, microtubules of larger diameter with 14 and 15 protofilaments are also found in some species, for example, in the nematode *Caenorhabditis elegans* (*C. elegans*), where 15-protofilament microtubules exist in six of the touch receptor neurons [6]. Also in *C. elegans*, microtubules with a smaller diameter corresponding to 11 protofilaments are found in the nerve chord and intestine [7, 8]. This suggests that microtubules may vary their structures to potentially specify different functions. Generally, microtubules assembled in vitro from pure tubulin (usually mixed-isoform brain tubulin) have between 9 and 16 protofilaments, with 13 and 14 being the most abundant depending on the assembly conditions used [9–11]. Microtubule protofilament number can be modulated by various substances including different solvents [12], slowly-hydrolysable nucleotides [13], microtubule-targeting drugs [14] and MAPs [14, 15]. Further, protofilament numbers and seam numbers along the same microtubules can vary for microtubules assembled in vitro from purified brain tubulin [16, 17]. Transitions of protofilament numbers indicate that microtubule lattices are plastic.

Why do mammalian cells usually prefer a 13-protofilament lattice? Perhaps “straight-protofilaments” is the answer to this question as microtubules with 13 protofilaments have their protofilaments aligned parallel to the long axis of microtubules, providing linear tracks for molecular motors to walk along [18], for example, the cargo-bearing kinesin-1 motor can walk upwards of a micron along
a single protofilament [19]. In comparison, protofilaments in other commonly found arrangements adopt twisted superhelical configurations, with helical pitch typically ranging from 4 to 6µm, that wind around the microtubule axis [19–21]. This would force protofilament-tracking molecular motors to spiral around microtubules, posing challenges for directional organelle transport in dense cellular environments, such as transport of mitochondria and vesicles in axons [18]. Whether this truly generates selective pressure remains unclear as some kinesins do not walk along just one single protofilament, instead, they can drift or wander [22]. In addition, protofilament numbers can change at different axial positions, generating “defects” in microtubules in cells [9]. This raises a question whether “defects” are actually essential structural features that ensure organisms can carry out certain physiological functions.

In cells, microtubules prefer a pseudohelical structure with a seam breaking their symmetry. Seams are due to the formation of an atypical stagger between two neighbouring protofilaments. This so-called A-lattice seam has α-β tubulin lateral contacts, see Figure 1.1. Away from this A-lattice seam, the other protofilaments associate via homotypic α-α and β-β tubulin lateral contacts, forming the so-called B-lattice. As a result, 13-protofilament microtubules normally have a 3-start configuration (usually referred to as 13-3, protofilament number-helix start number) in which the protofilaments flanking the seam are staggered by 4.9 nm whilst the rest of the protofilaments make B-lattice contacts, whereby the neighbours stagger by 0.9 nm [23]. Microtubules formed in vitro can have different helix start numbers ranging from 2 to 4, but these arrangements are thought to be less favourable as they cause protofilaments to skew steeply away from the microtubule axis [24].

The implications of the broken helical symmetry caused by the A-lattice seam are unclear. The seam could possibly make microtubules more stable since 13 protofilaments with a seam were favoured and selected by evolution [25]. However, there is conflicting evidence on whether the A or the B lattice is stronger. The interprotofilament interactions in the seam region were thought to be similar to those in normal B-lattice helical regions based on MD simula-
Figure 1.1: Microtubule lattice arrangement. A typical microtubule lattice found in vivo consists of a 3-start helix B-lattice with an A-lattice seam.

On the contrary, more recent molecular dynamics simulations [27] suggest that the A-lattice is less stable than the B-lattice. In addition, early experimental data showed that microtubules unzip along a single cold-sensitive seam after cold-shock to become an open sheet, implying that the A-lattice might serve as a hotspot for microtubules to unzip and depolymerise [28]. Consistent with this idea, microtubules with multiple ectopic A-lattice seams induced by Mal3 have been shown to be highly unstable [29]. However, interestingly, EB3 (end-binding protein, a human Mal3 ortholog) was found to promote GMPCPP (slowly-hydrolysable GTP analog) hydrolysis, thereby destabilising the lattice [30]. Nevertheless, the weaker seam theory is also supported by more recent structural data where both 13- and 14-protofilament GDP microtubules have a larger seam opening than those of GMPCPP microtubules, which may serve as a hotspot for microtubule unzipping [31].

Other than existing as a single tube, doublet and triplet microtubules with various protofilament arrangements are also found. Microtubule doublets, each of which consists of an A-tubule and a B-tubule, are commonly observed...
in cilia and flagella whereas triplets are only found in centrioles. Centrioles are composed of long-lived microtubules organised as nine triplets in most organisms, including humans [32]. This triplet structure is made up of a complete A-tubule with a partial B-tubule attached to the A-tubule and a partial C-tubule adjacent to the B-tubule [33, 34]. The A-tubule consists of 13 protofilaments whereas B- and C-tubules are each made up of 10 protofilaments, regardless of doublet or triplet microtubules. Despite the well-known mammalian somatic centriole cycle, the mechanism by which microtubules are made into triplets remains elusive [32].

Due to the heterodimer packing, microtubules have polarity, so that their ends possess distinct properties. The (slow growing) minus and (fast growing) plus ends have terminal α and β tubulins, respectively. The polarity signposts cargo-bound molecular motors to walk along unidirectionally to transport intracellular materials to the correct destination. For example, in axons, microtubules have a uniform arrangement with the plus end pointing away from the cell body whilst dendrites have a mixed polarity, each of which favours particular post-translational modifications (PTMs) such as acetylation or tyrosination [35–39].

1.1.1 Microtubules show dynamic instability

At steady state, microtubules undergo alternate phases of steady growth and sudden shrinkage. This behaviour is known as microtubule dynamic instability. The transitions from growth to shrinkage and the reverse are termed catastrophe and rescue, respectively. Dynamic instability is stochastic and is believed to facilitate regulation of cell activities such as cell division and migration via reorganisation of microtubule arrays. Another elusive behaviour of microtubules is treadmilling, where the microtubule plus end constantly grows as the minus end continues to shrink. This phenomenon has been observed in cells but to a lesser extent in vitro with pure tubulin [40, 41].

Both plus and minus ends of microtubules show dynamicity in vitro.
which can be defined by their distinct dynamics with the faster growing end being the plus end. However, the minus end is usually not dynamics in vivo as microtubules often emerge from \( \gamma \)-tubulin ring complex (\( \gamma \)-TuRC) which serves as a nucleation site for a single microtubule. Whilst catastrophe is more likely to happen at the plus end, rescue is more frequent at the minus end [42]. Microtubule dynamics can thus be characterised by several parameters, including growth and shrinkage rates, and frequency of catastrophe and rescue, at the plus and minus ends. These parameters can be tuned by salts, nucleotides and other proteins.

Microtubule growth rates are directly proportional to free tubulin concentration. Although depolymerisation rates are generally thought to be independent of the concentration of soluble tubulin, this view has been challenged [43]. Other than growth rate, free tubulin concentration also modulates other microtubule properties such as catastrophe and rescue frequencies. While catastrophe frequency decreases with increased tubulin concentration for both microtubule plus and minus ends, rescue frequency is directly proportional to tubulin concentration, albeit the plus end is less sensitive to free tubulin concentration compared to the minus end [42]. The view that catastrophe is purely stochastic has been challenged, as microtubule plus end lifetimes do not simply follow an exponential distribution. Rather, a gamma distribution provides a better fit, suggesting catastrophe could be a multi-step process potentially associated with microtubule age [44, 45].

1.1.1.1 Tubulin conformations are coupled to GTP turnover

The interplay between tubulin conformations and nucleotide states determines whether tubulin dimers can assemble into microtubules. Tubulin is assembly incompetent in the absence of GTP unless facilitated by drugs such as taxol [46]. Both \( \alpha \) and \( \beta \) tubulin monomers have nucleotide binding sites which allow binding of GTP molecules. GTP buried in the so-called non-exchangeable site (N-site) of \( \alpha \) tubulin is neither hydrolysable nor exchangeable whereas GTP
in the exchangeable site (E-site) of \( \beta \) tubulin can be hydrolysed to GDP and exchanged with GTP in solution. GTP turnover in the E-site is generally required to drive microtubule assembly, although several drugs, such as taxol, can overcome energy barriers required for tubulin association and drive assembly of GDP-tubulin in the absence of GTP [46].

Early research favoured the view that GDP-tubulin is intrinsically bent [47], largely due to the fact that electron micrographs showed depolymerising microtubule tips having a frayed or ram’s horn structure. Classically, loss of the GTP-tubulin cap triggers catastrophe by exposing the unstable microtubule shaft, with instability, originating in the drive for GDP-tubulin to bend outwards, causing the entire microtubule to unzip [48]. The curvature of GDP-tubulin protofilaments is clear but it is less clear whether GTP-tubulin protofilaments also curve. Unfortunately, the conformation of free GTP-tubulin in solution is still an enigma, whereas it is widely-accepted that free GDP-tubulin is intrinsically bent and therefore strained and unstable when packed into straight, parallel protofilaments in microtubules. Recent cryo-electron tomography data suggest that protofilaments of growing microtubules also adopt a curved conformation at their tips which could be an indication of an intrinsically bent GTP-tubulin conformation [49]. Structural data other than cryo-EM also suggest that GTP-tubulin adopt a bent conformation in solution either by small-angle x-ray scattering or using GTP-tubulin complexed with a stathmin-like domain for crystallography [50, 51]. Although it is generally accepted that catastrophe is a result of loss of the “GTP cap”, rescue, whereby depolymerisation is interrupted and growth resumes, remains poorly understood.

1.1.1.2 The detailed mechanisms of microtubule assembly remain controversial

Also elusive is the mechanism by which microtubules form in vitro. Microtubules form only when the concentration of free tubulin in solution exceeds a critical value – critical concentration. Microtubules were believed to begin to as-
semble (nucleate) when short protofilaments which come into contact with each other, bind laterally to form a sheet-like structure. This sheet then closes when the first and last protofilaments meet each other, forming a closed tube. A similar tube closure process was originally thought to account for microtubule elongation [25, 52]. More recent structural data provide deeper insights into nucleation, suggesting microtubule assembly starts from single stranded tubulin oligomers, with relatively low curvatures and a particular size (number of tubulin dimers). These “straighter” single stranded tubulin oligomers have a higher tendency to form multi-stranded oligomers as the straight conformation is more thermodynamically favourable for establishment of lateral contact. Multi-stranded oligomers then eventually come together to form short microtubules (seeds) [53]. After seed formation, microtubules grow via the addition of bent GTP-tubulin dimers to the tips as suggested by demonstration of flared protofilaments of growing microtubule ends by cryo-electron tomography, which eventually come together to form a tube [49]. Therefore, microtubule ends are often capped by GTP-tubulin [54]. Interestingly, microtubules with a longer “GTP cap”, corresponding to faster growth rate, tend to have lower catastrophe frequency.

1.1.1.3 Microtubule dynamic instability in vivo is heavily regulated by interactors

The growth and shrinkage reactions of microtubules in vivo are more complicated than those in vitro, because microtubules interact with various proteins called microtubule-associated proteins (MAPs). These MAPs have crucial roles as they not only regulate microtubule dynamics, acting as polymerases and depolymerases, but also microtubule conformations, as discussed in more details in Section 1.5.3. For example, a type of kinesin-13, mitotic centromere-associated kinesin (MCAK), accelerates microtubule depolymerisation [55] whereas Mal3, an EB protein, suppresses catastrophe [56].

Unlike spontaneous formation of microtubules in free solution in vitro; in cells, microtubules often emerge from a microtubule-organising centre (MTOC).
MTOCs are assembled mainly from γ-TuRC, which is made up predominantly from 14 γ-tubulin molecules, “capping” individual microtubule minus ends. However, microtubules in cells emerge from γ-TuRCs usually have 13 protofilaments [57].

1.2 Tubulins have 3 major domains

α and β tubulin subunits have similar structures: a core structure linked to a C-terminal tail. The core consists of three major domains: N-terminal (N), intermediate (I) and C-terminal (C) domains linked to the disordered C-terminal tail (CTT) [58, 59], see Figure 1.2(a). Some parts of tubulin are not to date resolved by EM due to their disordered properties, such as the acetylation loop of α tubulin. In addition, the CTTs of both α and β tubulins, which are exposed on the microtubule surface, are freely fluctuating, so they do not yield a clear structure.

These three major domains provide interaction sites for tubulin dimers as well as for MAPs. The N-domain harbours the GTP molecule. Part of the N-domain of β tubulin is exposed at the plus end whereas the I-domain of α tubulin makes up most of the minus end. The intradimer interaction of individual α and β tubulin monomers involves the N-domain of α tubulin and I-domain of β tubulin in head-to-tail fashion. Similarly, microtubule protofilaments elongate via the interdimer interaction of N-domain in β tubulin of microtubule tip and I-domain in α tubulin in solution.

The stability of tubulin dimers can be explained by the tight interaction between α and β subunits, with the GTP-binding site of α tubulin buried at the intradimer interface. The GTP in the N-site in α tubulin cannot be exchanged or hydrolysed [59], see Figure 1.2(b). On the contrary, in microtubule tip region, GTP in the E-site in β tubulin can be hydrolysed by the catalytic glutamate of α tubulin (α-E254) of longitudinally adjoining tubulin dimer.
Figure 1.2: Tubulin domains and GTP binding sites. (a): Individual tubulin monomer consists of three major domains: N-terminal (yellow), intermediate (magenta) and C-terminal (cyan) domains. CTTs are not resolved by cryo-EM. K/D: kinesin and dynein binding site; DC/EB: doublecortin and EB binding site (binding site not drawn to scale). PDB: 5JCO. (b): Lateral view of tubulin dimer with polarity marked. GTP buried in α tubulin is non-exchangeable and non-hydrolysable whereas the nucleotide bound in β tubulin is exchangeable and can be hydrolysed. The C-termini of H12 helices (labelled as H12) of respective tubulin subunits are connected to CTTs which are not resolved by EM.
In addition to longitudinal interactions, N- and I-domains are also involved in lateral interactions between protofilaments. Interdimer lateral interactions mainly require participation of the M-loop of one tubulin with the H1-S2 and H2-S3 loops of another tubulin [58]. Binding of some MAPs predominantly involves the C-domain, made up of two helices, and the negatively-charged CTT, which is enriched in acidic residues [58]. The roles of CTTs in MAP interactions have been demonstrated by microtubules that have had their C-termini cleaved off by subtilisin. These microtubules show a diminished landing rates and shorter run lengths for kinesin and dynein [60]. Therefore, changes in these motifs, be it divergent residues or PTMs, may regulate microtubule properties.

Interestingly, microtubule structures and properties are sensitive to some particular single residue modulations. Multiple residues in both α and β tubulins have also been identified to play crucial roles, for example, α-K40 is subject to acetylation to regulate lateral interaction between protofilaments [61]. Mutagenesis of the α-E254 residue to alanine can block GTP hydrolysis in microtubule lattice, resulting in hyperstable microtubules [62]. The role of this residue in single isoform microtubules will be further discussed in Section 1.9.

1.3 The multi-tubulin hypothesis

Sequences of α tubulin isoforms are more self-similar than β tubulin isoforms. The core of vertebrate tubulin is approximately 400 amino acids in length, having about 97% and 95% sequence similarity for α and β tubulins, respectively [63]. In contrast, CTTs are the most divergent regions with only about 50%–60% similarity [64, 65]. These CTTs undergo extensive PTMs [66], perhaps to increase tubulin functional diversity, for example, by recruiting different interactors.

In general the more complex the organism, the greater the diversity of tubulin isoforms. In humans, there are 8 α and 10 β tubulin isoforms, see Figure 1.3. Mice have 7 α and 8 β tubulin genes [67]. In another commonly used
model organism, the fruit fly *Drosophila melanogaster* (*D. melanogaster*), 4 α and 4 β tubulin genes have been identified. Fission yeast *Schizosaccharomyces pombe* (*S. pombe*) has only 2 α and 1 β tubulin genes. The great diversity of tubulin isoforms in more complex organisms suggests the possibility that different tubulin isoforms specify different functions.

Despite high sequence similarity, some tubulin isoforms are not interchangeable [68], suggesting the critical roles of certain tubulin isoforms. For example, α1 tubulin gene (*nda2*) in fission yeast *S. pombe* is indispensable whereas α2 tubulin gene (*atb2*) is nonessential [69]. In addition, even a single point mutation in one tubulin isoform can cause tubulinopathy – typically neuropathy [70].

To further support the idea that specific tubulin isoforms may play different roles in organisms, some tubulin isoforms have been found to express exclusively in particular tissues. For example, β3 tubulin is ordinarily specific to neurons. Its non-neuronal expression is often associated with some cancers [71]. β1 tubulin (encoded by gene *TUBB1*, may also be referred to as βVI tubulin, not to be confused with class I β tubulin which is sometimes referred to as βI tubulin) is highly expressed in platelets and its mutation is linked to blood disorders [72, 73].

On the other hand, some other tubulin isoforms have more global expression, potentially having roles in fundamental cell activities. As a result, most tissues and organs normally express a mixture of tubulin isoforms. Bovine and porcine brains are perhaps the most widely-used sources of tubulin and the most well-characterised ones. Class II β tubulin makes up 58% of total β tubulin in the brain, with 25% of class III (Gene: *TUBB3*) tubulin as the second abundant β tubulin component, in addition to 3% of class I (Gene: *TUBB*) and 13% of class IV (Gene: *TUBB4A* or *TUBB4B*) tubulin [71].

Among the highly self-similar α tubulins, α4a tubulin, which is highly expressed in platelet, and α8 tubulin, are the unique ones as the gene-encoded terminal tyrosine residue present in the CTTs of other α tubulins are lacking
Figure 1.3: Sequence alignment of known human α and β tubulins. The conserved residues of α (top) and β tubulins (bottom) are shown in the darkest shade. Two selected conserved catalytic residues, E254 and E415, of α tubulins which are responsible for nucleotide hydrolysis of GTP in tubulin and ATP in kinesin are also shown.
in these two isoforms, see Figure 1.3. Interestingly, some α tubulins undergo
detyrosination and retyrosination, emphasising the roles of particular residues as
well as PTMs specific to these residues.

Despite our knowledge about the sequences of these tubulin isoforms,
the specific roles of many of these tubulin isoforms still remain elusive. It is
currently unclear whether certain α and β tubulins preferentially dimerise with
each other. The uncertainty is due largely to the unavailability of antibodies
specific to the individual isoforms, especially for α tubulins [74]. Hence, there
is a need to elucidate the roles of each tubulin isoforms in vitro, to generate
hypotheses that can hopefully be tested in vivo in the near future.

1.3.1 Single residue mutations in particular tubulin isoforms can pro-
duce serious disease

Since microtubules have multiple roles in metazoan development and
growth, mutations in tubulin encoding genes can cause a series of developmental
brain disorders [70] and other diseases. In addition, due to the exclusiveness of
some tubulin isoforms in particular tissues, point mutations at important sites
could cause perturbations of tissue-specific microtubule functions and provoke
disorders. In humans, mutations in some isoforms, even with just one residue
substitution, cannot be rescued by another tubulin isoform. For example, R307H
residue substitution in β1 tubulin affects microtubule dynamics and causes hered-
itary thrombocytopenia, since β1 tubulin is enriched in platelets [75]. Mutation
of one residue in another β tubulin isoform, encoded by gene TUBA3D, has been
linked to keratoconus [76].

Due to the presence of microtubules in almost all eukaryotic cells, de-
fects in organelles with abundant microtubules may cause systemic effects, for
example, ciliopathy - primary ciliary dyskinesia (PCD), is associated with situs
inversus and bronchitis. Furthermore, PCD might also lead to infertility as flag-
ellar motility of sperms relies on the sliding of doublet microtubules against each
other [77]. Thus, mutations in tubulins may result in sperm immotility or low motility [78].

In addition, perturbations in other microtubule-relevant activities, such as PTMs and other MAP regulated activities, can also cause a spectrum of diseases. For example, impaired microtubule dependent vesicle transport, one of the hallmarks of Huntington’s disease, is associated with aberrant tubulin acetylation [79].

1.4 Post-translational modifications of tubulin regulate microtubule activities

Tubulins undergo various post-translational modifications (PTMs), potentially regulating a myriad of microtubule activities and functions. Both free tubulin and polymerised tubulin can undergo PTMs with the majority occurring to the CTT, especially for $\alpha$ tubulin in microtubule lattices [80]. Due to the locations of CTTs on the surface of microtubules, PTMs of CTTs could potentially regulate binding of MAPs [81].

While tyrosination is exclusive to tubulin, few other PTMs such as (poly)glycylaion and (poly)glutamylation use substrates in addition to tubulin [82, 83]. Certain PTMs are observed more frequently in particular tissues, for example, glycylaion is thought to occur more often on cilia and flagella in mammalian cells [84]. Relatedly, perturbations of PTMs could potentially lead to diseases such as ciliopathies [85, 86] and neurodegeneration [87].

Perhaps tubulin isoforms may specify PTMs, especially when most types of PTMs are found on the highly divergent CTTs, which might serve as recognition sites for respective enzymes.
1.4.1 Post-translational modifications on C-terminal tails

Of all the PTMs on CTTs, tyrosination/detyrosination are amongst the most interesting ones as they have yet to be found in proteins other than α tubulin [82, 88]. Tyrosination is a reversible process which can happen following removal of the gene-encoded C-terminal tyrosine residue (detyrosination) of α tubulin [89]. Detyrosination can be followed by irreversible removal of the penultimate glutamate to give rise to Δ2-tubulin which can also trigger further removal of an additional amino acid to become Δ3-tubulin [90, 91]. Detyrosination has been demonstrated to suppress microtubule depolymerisation by kinesin-13 such as KIF2C, also known as MCAK, and KIF2A [92].

In contrast, (poly)glutamylation and (poly)glycylation can modify both α and β tubulins, resulting in branching of CTTs whereby additional glutamate or glycine chains are added to gene-encoded glutamate residues in the CTTs by tubulin tyrosine ligase-like (TTLL) glutamylases and glycylases, respectively [93, 94]. Polyglutamylation has been found to occur preferentially on residue 445 and 435 in α and β tubulins, respectively, although other glutamates nearby can also act as substrates [93, 94]. Interestingly, the length of glutamate chain can potentially specify microtubule functions as glutamate chains ranging from 1 to 6 glutamate molecules are commonly seen in neurons whereas more than 10 glutamates per chain are found in cilia [93, 95, 96]. These two PTMs may modulate different cell activities. For example, polyglutamylation has been found to regulate activities of some MAPs, such as tau [97] and kinesin-1 [63] as well as microtubule severing activities [98]. In contrast, polyglycylation is commonly found in axonemes and affects the length and stability of cilia [99, 100]. Unlike polyglutamylation [101], polyglycylation is not yet known to be reversible.
1.4.2 Post-translational modifications on tubulin core

Fewer PTMs have been identified on the tubulin core than on the CTTs of tubulins. The known ones are acetylation, polymamination and phosphorylation with acetylation perhaps being the most intriguing one as it is the only PTM found on the surface of the microtubule lumen.

Acetylation of α-K40 by αTAT1 (tubulin acetyl transferase, which prefers tubulin in the microtubule lattice over free tubulin), is one of the PTMs commonly found in cilia and flagella [102]. Acetylation has been demonstrated to confer microtubule stability by weakening the lateral interactions between protofilaments, reducing mechanical stiffness [61, 103]. Consistent with weakened interprotofilament interactions, acetylated microtubules depolymerise faster than non-acetylated ones, but these seemingly less stable microtubules are in fact more long-lived, possibly due to enhanced microtubule flexibility, thereby improving resistance to mechanical stress [103]. Although well-known for modification of K40 in α tubulin, acetylation of β tubulin has also been found [104] on K252 where the reaction is carried out by a different enzyme, the acetyltransferase San. Acetylation of β-K252 is thought to regulate microtubule polymerisation [105].

Other than regulating the microtubule lattice, acetylation of microtubules may also regulate microtubule interactions with some effectors. Acetylation may promote binding of kinesin-1 to microtubules, albeit some kinesins are not biased towards acetylation, including kinesin-2 and kinesin-3 [106, 107]. In addition, absence of acetylation in fibroblasts derived from αTAT1 knockout mice rendered microtubules more resistant to nocodazole-induced depolymerisation [108] which suggests potential association of acetylation with drugs. Furthermore, αTAT1 knockout mice have reduced sperm motility, hence, decreased fertility [103, 108], linking PTMs of tubulin to normal cell functions.

Phosphorylation, being one of the most ubiquitous PTMs in cells, can also modify tubulins. This reaction is believed to regulate microtubule engage-
ment with cell membranes and microtubule polymerisation [109]. Polyaminated microtubules, which are cold-resistant and more basic in charge, are often found in the axons of neuronal cells and are enriched in testes whilst being scarce in other non-neuronal cells [110, 111].

1.5 Allosteric effectors can powerfully influence microtubule properties

Other than modifications of residues in tubulin, microtubule structure and dynamics can be regulated through signal transmission from one region to another or by other effectors such as nucleotides and microtubule-associated proteins (MAPs). For example, addition and loss of tubulin subunits at microtubule tips are both catalysed, by polymerases and depolymerases, respectively [55, 112].

MAPs can be divided into two major categories: motor proteins and non-motor proteins. The motor proteins kinesin and dynein have overlapping binding sites bridging the tubulin intradimer interface [113], see Figure 1.2(a) for main MAP binding sites. Other MAPs such as doublecortin and end-binding (EB) proteins bind between protofilaments, at the interface of 4 tubulin dimers with $2 \times 2$ arrangement. Doublecortin avoids seam binding [114, 115].

MAPs regulate microtubules in various ways such as lattice stabilisation and microtubule length regulation. Just as MAPs regulate microtubules, so can microtubules regulate MAP activities, for example, microtubules accelerate ATP hydrolysis and ADP release in kinesin, thereby coupling ATP turnover to kinesin stepping.
1.5.1 The lattice as an allosteric effector

Tubulin molecules in the lattice may sense and influence the conformation of their neighbours. Kinesin-1 confers microtubule stabilisation in the absence of free tubulin (microtubules would ordinarily depolymerise in the absence of free tubulin) as well as microtubule lattice expansion. For both activities, full decoration of microtubules by kinesin is not required, especially with rigor kinesin [116]. Peet and colleagues demonstrated that microtubules clamped down by kinesin-1 decorated coverslips depolymerised slower in the absence of nucleotide (kinesin in rigor state) compared to that in the presence of ADP (weak binding state) [116]. This suggests that with just a maximum of 5 protofilaments bound to kinesin (due to geometry constraint), is sufficient to stabilise the entire microtubule, potentially due to signal transmission laterally around microtubule axis [116].

In addition, Shima and coworkers have also demonstrated co-existing GDP microtubules with different extent of kinesin decoration at substoichiometric concentrations of kinesin-1 [117]. They also showed by X-ray fibre diffraction that at a 5% kinesin/tubulin concentration ratio, microtubules exhibited mixed populations of compact and expanded lattice whereas at a 10% concentration ratio, most microtubules adopted the expanded lattice. Both these findings suggest cooperative lattice expansion and cooperative binding, whereby neighbouring tubulins detect each other’s conformation and respond accordingly, allowing allosteric effectors to elicit concerted conformational changes.

1.5.2 Conformational changes caused by GTP hydrolysis affect microtubule structure and function

Conformational changes induced by GTP hydrolysis, which can sometimes be modulated by MAPs, are inherent in microtubules. Upon tubulin dimer addition to the tip of a growing microtubule protofilament, GTP in the E-site in microtubule lattice is hydrolysed, typically accompanied by lattice compaction.
The expanded GTP microtubule lattice was previously demonstrated using microtubules assembled in GMPCPP, a slowly-hydrolysable GTP analog, under cryo-EM, compared to dynamic GDP microtubules [30, 118]. GTPγS, another slowly-hydrolysable GTP analog, has been reported to generate microtubules with a compact lattice [31]. GTPγS is also incapable of promoting tubulin nucleation [119]. Therefore, GTPγS microtubules are believed to mimic microtubules in the GDP.Pi state, whereas GMPCPP microtubules are thought to be more GTP-like.

Advances in cryo-EM have allowed structural studies of microtubules with different nucleotide states or different MAPs bound to be seen in finer detail. The majority of structural information reported to date had microtubules labelled with kinesin or other MAPs as fiducial markers to distinguish α and β tubulins, which otherwise have highly similar structures. GDP pig brain microtubules have the shortest dimer repeat at 81.5 Å whereas GMPCPP microtubules have the longest at 83.2 Å [30, 118], both of which determined with the use of kinesin molecules as markers. With more recent cryo-EM algorithms and detectors, marker free GMPCPP pig brain microtubule dimer rise was reported to be 83.95 Å whereas the kinesin-1 decorated microtubules had a slight compaction giving 83.30 Å [31]. The possibility of using marker free microtubules has revolutionised microtubule structural studies, as MAPs used as fiducial markers can potentially cause conformational changes to microtubules which can be misleading sometimes for studies of the intrinsic structural dynamics of microtubules.

Despite the fact that GTP hydrolysis is often associated with microtubule compaction, this is not always so. Dynamic yeast microtubules, when assembled in GTP, do not compact following hydrolysis, though they do show robust dynamic instability, as shown in S. pombe [114] and Saccharomyces cerevisiae (S. cerevisiae) [120] studies. On the other hand, S. cerevisiae microtubule does compact upon GTP hydrolysis, but only in the presence of Bim1, a S. cerevisiae EB homolog [120]. Interestingly, S. cerevisiae microtubules in GTPγS (generally thought to be mimick a GDP.Pi state) is slightly compacted, with the most compaction found in GTPγS microtubules with Bim1 bound [120].

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suggests a diversity of microtubule structural response to even the most common and fundamental microtubule process, namely GTP hydrolysis.

Although it is easy to relate the instability of microtubules, in their GDP state, to the strain energy stored in individual GDP-tubulin dimers that are “forced” into relatively straight conformation by assembly into microtubules, the detailed mechanism by which GTP hydrolysis leads to microtubule catastrophe still remains elusive. This hot topic has been partially elucidated by recent work using doublecortin-bound microtubules in different nucleotide states. Structural changes of microtubules upon GTP hydrolysis were proposed to happen in two stages: firstly a loosening of lateral contacts between α-α tubulin, followed by a weakening of β-β tubulin lateral contacts. These transitions involve interplay between lateral and longitudinal interdimer interactions of tubulin as these two types of contacts undergo seemingly counteracting movements. Surprisingly, α tubulin undergoes major conformational changes upon GTP hydrolysis, instead of β tubulin which is the site where hydrolysis happens [30, 115]. These changes in α tubulin triggered by GTP hydrolysis causes uneven compression of α tubulin which leads to the striking reinforcement of longitudinal interdimer interactions. Despite this seemingly stronger interdimer associations, GDP microtubules remain subject to catastrophe due to their weakened lateral contacts, exacerbated by the uneven α tubulin compression. The accompanying strain leads to failure of the attenuated lateral contacts, eventually leading to microtubule catastrophe [115].

Recent data using single isoform tubulin mutants which are GTP hydrolysis incompetent provide new insight into microtubule plasticity. This work used two seemingly similar GTP microtubule mutants which only differed in one residue that is critical for GTP hydrolysis. The catalytically inactive mutant microtubules both adopt an expanded lattice, as expected based on GMPCPP microtubule structure, however, one mutant has positive lattice twist whereas another mutant can conform to either positive or negative skews [4]. Strikingly, EB3 can compact these microtubules despite the presence of GTP in β tubulin [4], raising more questions about the relationship between GTP and tubulin
Crucially, whilst lattice compaction following GTP hydrolysis appears variable, the fundamentals of dynamic instability (GTP lattice stable, GDP lattice unstable) are consistent, indicating that a conformational change is occurring, but does not necessarily result in a spacing change.

1.5.3 Microtubule regulation by microtubule-associated proteins

Both motor and non-motor microtubule-associated protein (MAPs) can regulate microtubule activities or microtubule conformations or both. Some MAPs can recognise specific microtubule conformations or nucleotide states. For example, EB, which is one of the microtubule plus-end tracking proteins (+TIPs), binds preferentially at the plus end. EB is a protein family which consists of EB1, EB2 and EB3. EB yeast homolog Mal3, not only regulates microtubule structure by generation of multiple seams but also microtubule dynamics through increase in rescue frequency [15]. In addition to the effects on individual microtubules, some MAPs act as cytoskeletal crosslinkers to bundle microtubules whilst allowing microtubule sliding against each other, such as kinesin-14 (Ncd) which controls sliding of anti-parallel microtubules but cross-links (statically) parallel microtubules of spindle microtubules during mitosis [121].

The motor proteins, as the name suggests, act like “engines” to move along microtubules to transport intracellular materials as well as to regulate various activities. Kinesin superfamily members are involved in vesicle transport and regulation of microtubule dynamics in both dendrites and axons [122]. Kinesins form a diverse protein superfamily which can be categorised into different ways such as stabilising/destabilising kinesin, plus/minus end directed kinesin or the N-/C- terminal motor domain. Although most kinesin family members are plus-end directed, there are exceptions such as kinesin-14 which is minus-end directed [123], and strikingly, some kinesins can switch direction while tracking along microtubules such as kinesin-5 [124, 125].
Most MAPs, including kinesins, regulate microtubules by causing tubulin conformational changes in the lattice. Whilst kinesin-13 destabilises microtubules by bending tubulin or stabilising a bent conformation of tubulin [126], kinesin-1 stabilises microtubules accompanied by lattice expansion [116, 117]. In addition, some kinesins are highly processive so can walk along microtubules for a long distance as a single molecule, like kinesin-1 [127] whereas kinesin-14 is non-processive as a single molecule [128].

Recently, a further different group of MAPs has been identified – the microtubule inner proteins (MIPs), found in ciliary doublet microtubules, which are believed to be critical for structures such as connecting A- and B-tubules or to resist mechanical stress induced by ciliary movement [129]. This discovery raises new questions about the roles of MIPs as they are “hidden” in microtubules and how they get incorporated into microtubule lumen.

1.5.3.1 Conserved and divergent residues in tubulin isoforms govern generic and specific functions of microtubules

Due to the fact that pure single isoform human tubulins have only become available in recent years, microtubule-MAP interactions have mainly been studied using brain microtubules or yeast microtubules. The availability of defined-isoform tubulins now makes it possible to dissect the enigmatic two-way interactions between MAPs and single isoform microtubules. Ti and colleagues demonstrated whilst MCAK and chTOG, bound to a similar extent to GMPCPP $\alpha_{1b}/\beta_{2b}$ and $\alpha_{1b}/\beta_{3}$ microtubules, as determined by fluorescence intensity, the $\alpha_{1b}/\beta_{3}$ microtubules still depolymerised faster by 5 to 9 fold, depending on the MAP and experimental conditions used. This suggests that $\alpha_{1b}/\beta_{3}$ microtubules are intrinsically less stable [3]. In addition, when the growth speeds of mouse brain microtubules and $\alpha_{1b}/\beta_{I+IVb}$ were adjusted to be similar, EB1 comet lengths at the plus ends of both types of microtubules were comparable [130]. This finding potentially suggests that the GTP hydrolysis rate in microtubules is rather conserved, depends primarily on growth rate and is perhaps independent
of the tubulin isoform. However, a caveat of this study is that brain tubulin, as reported by Banerjee and colleagues, is composed of 13% of β4 tubulin [71], so the distinction between brain microtubules and α1b/βI+βIVb microtubules might have been blurred. All these findings do tend to indicate that at least some MAPs do not discriminate tubulin isoforms, perhaps to ensure generic microtubule functions. Also, as discussed in Section 1.9, the single isoform GTP hydrolysis-incompetent β3 tubulin mutant E245N assembled into microtubules with distinct lattices that result in differential EB binding, raising question about this recognition mechanism.

Another study with the use of yeast S. cerevisiae expressed a synthetic chimeric tubulin composed of a yeast tubulin core and respective human CTTs, for investigation of the roles of CTTs in interactions with kinesin-1, kinesin-2 and dynein [63]. The authors found that the chimeric β1 and β3 microtubules have reduced kinesin-1 run length, possibly due to the presence of a positively charged lysine at the C-terminus. Relatedly, the β3-Δlysine mutant restored kinesin-1 run length [63]. All these findings highlight the contributions of CTTs for specific MAP regulations, albeit the tubulin used was not fully representative of human version.

The sensitivity of microtubule dynamics to MAPs, and the sensitivity of MAP binding to small differences in microtubule structure, will tend to amplify the effects of tubulin mutations. Microtubules assembled from tubulin with pure human β3 tubulin mutants R262H or D417H, expressed in insect cells, showed significantly lower kinesin-1 and PRC1 affinities [131]. These two residues are conserved in all human β tubulin isoforms, underlined the involvements of several important residues in interactions with some MAPs to govern normal microtubule functions and activities. The human α tubulin component in these experiments was untagged so the resulting microtubules had a certain level of insect α tubulin contamination.
1.6 Microtubule drugs have different binding sites and distinct mechanisms

Microtubule-targeting agents (MTAs), some of which have been used for cancer treatments, can be divided into two major categories: microtubule-destabilising agents (MDAs) and microtubule-stabilising agents (MSAs). Interestingly, most MTAs that have been identified so far bind β tubulin, see Figure 1.4.

Figure 1.4: Microtubule-targeting agents and their respective binding sites. Figure adapted from Steinmetz & Prota, 2018 [132].

As the names suggest, these drugs should either make microtubules more stable or less stable. However, these two terms can sometimes be confusing as they typically refer only to the effects observed when the drugs are used at relatively high concentrations. When administered at (clinically-relevant) low concentration, both MDAs and MSAs inhibit microtubule dynamics without sig-
ificant effects on microtubule polymer mass [133].

Surprisingly, although microtubules exist in almost all cells, some drugs are actually more effective against particular cancers than the others. For example, Taxol® (generic name paclitaxel) is prescribed for breast, ovarian and lung cancers [134, 135] whereas vinca alkaloids are normally used for haematological cancers. This raises a question why these drugs are biased towards some cancers when the target “microtubules” play overlapping roles such as cell division in organisms.

Although some of these drugs are effective against particular cancers, off-target effects, such as neuropathy and myelosuppression, pose challenges for prescription and treatment outcomes. Thus, there is an urgent need for drugs with higher specificity for particular tubulin isoforms that overexpress in particular cancers. In addition, MTA resistance has begun to emerge, associated with overexpression of some tubulin isoforms sometimes [136]. Both drug specificity and resistance issues might potentially be addressed by learning more about the molecular mechanisms of the drugs and the exact roles of specific tubulin isoforms.

1.6.1 Microtubule-stabilising agents (MSAs)

Taxanes, including taxol and docetaxel; epothilones, such as epothilone A and epothilone B; in addition to laulimalide, are examples of microtubule-stabilising agents which bind different parts of β tubulin. Taxane and epothilone bind near the M-loop, which is involved in lateral interactions of protofilaments, on the luminal side of microtubule. Although taxol and epothilone compete for a common binding site in mammalian β tubulin, only epothilone stabilises yeast microtubules. Another MSA, laulimalide, has a distinct binding site on the outside of microtubule [137].

Taxanes have low affinity to soluble tubulin and bind more readily to
microtubules. One of the commonly used taxanes – taxol, suppresses microtubule catastrophe, thereby increasing microtubule polymer mass when used at high concentration. In contrast, substoichiometric concentrations of taxol inhibit microtubule dynamics without significantly increasing polymer mass [138]. Furthermore, like GMPCPP, taxol lowers tubulin critical concentration; thus, promoting microtubule assembly at lower free tubulin concentration, in addition to biasing protofilament number.

1.6.2 Microtubule-destabilising agents (MDAs)

After the identification of colchicine as the first MTA, vinca alkaloids such as vinblastine and vincristine were approved (over half a century ago) for use in lymphomas and other tumour treatments. Although colchicine and vinblastine bind different sites, when used at low concentration, both suppress microtubule dynamics. At higher concentrations, microtubule depolymerising effects are observed [133]. However, despite its effects on microtubules, colchicine has yet to be used for cancer treatment.

Colchicine destabilises microtubules by keeping curved tubulin dimers from straightening [139]. Unlike taxol, which binds mainly microtubules, it is unlikely that free colchicine binds microtubule directly; instead, it complexes with tubulin dimers which then copolymerise into microtubules, thereby preventing effective microtubule elongation [133]. Therefore, microtubule polymerisation can be inhibited by substoichiometric concentrations of colchicine.

When bound to microtubules, vinblastine acts as a wedge at the interdimer interface to destabilise microtubules [140, 141]. At lower concentration, vinblastine binds mainly to the tips of microtubules and less to tubulin in the lattice; thus, only one or two molecules are required at the plus end to reduce dynamics [142]. Vinblastine is capable of binding free tubulin when used at higher concentration, causing conformational changes that promote self-association, especially of GDP state tubulin [143].
1.6.3 Resistance to MTAs

Mutations in tubulin [144], overexpression of particular tubulin isoforms [145] or overexpression of MAPs such as MCAK [146], have been associated with resistance to taxol and epothilone A. To make the situation more complex, in cells, P-glycoprotein (a member of the family of ATP binding cassette transporters), which is a membrane protein, is sometimes upregulated, thereby increasing the efflux of taxol and other chemotherapeutics and, in consequence, reduction in intracellular drug concentration [147].

1.7 Taxol and different tubulin isoforms

Taxol is an FDA approved drug for use in the treatment of lung and breast cancer patients. Taxol was initially derived from the bark of Taxus brevifolia (Pacific yew tree). Researchers have been striving for the semi- and total synthesis of taxol since its discovery due to low yield of isolation from this slow-growing plant.

Taxol has poor solubility in water so a vehicle, cremophor/ethanol, is required for clinical use, both of which can generate issues in patients. Taxol has off-target effects which can potentially cause myelosuppression, neutropenia and peripheral neuropathies whereas the vehicles can cause anaphylactic shock. Despite being one of the most commonly prescribed and effective drugs, patients may develop resistance to taxol. Overexpression of some tubulin isoforms, such as β3 tubulin, is associated with poor taxol treatment outcomes and cancer prognosis [148, 149].

Like other MTAs, taxol, when used at low concentration, inhibits microtubule dynamics without significantly increasing microtubule polymer mass. When used at high concentration, taxol not only increases microtubule polymer mass, but also bundles microtubules. Taxol is also a strong tubulin nucleator.
that reduces tubulin critical concentration (the minimal tubulin concentration required for microtubule assembly) and can assemble GDP-tubulin into microtubules in the absence of GTP [46]. In addition, taxol protects microtubules from external depolymerisation stimulants such as cold- and calcium-induced depolymerisation [46].

Previous study found the most divergent region between β tubulins of yew tree (of species *Taxus baccata*), WWSSB1 and WWSSB2, and β tubulin of human TUBB, lying in the S9-S10 loop, apart from the CTT region [150]. The S9-S10 loop and the M-loop form part of the taxol binding pocket [151], underlining the importance of residues in these regions.

Taxol also does not have significant effects on yeast tubulin, such as *S. cerevisiae* tubulin, despite an approximately 75% sequence similarity to metazoan tubulin. On the other hand, epothilone, a drug which has an overlapping binding site as taxol, does act on yeast tubulin. Mutation of 5 out of 124 divergent yeast *S. cerevisiae* β tubulin residues to the corresponding mammalian tubulin residues, confers taxol sensitivity to yeast tubulin [152], implicating the involvements of these residues in mediating the binding or actions of taxol. These residues are located in different tubulin domains that make up taxol binding pocket in β tubulin, with three amino acids found in the N-domain (first letters indicating yeast residue and the last letter representing the mammalian residues): A19K, T23V, G26D, and the other two in the I-domain: N227H, and Y270F, see Figure 1.5. However, mutation of these residues in *S. pombe* tubulin does not introduce taxol sensitivity (Douglas Drummond, Cross lab, unpublished).

Other than yeast tubulin, β3 tubulin isoform has also been associated with taxol resistance which is potentially caused by a few divergent residues in β3 tubulin. For example, alanine 218 in the conserved leucine cluster (β212–β230) is replaced by threonine in other human β tubulin isoforms and has been suggested to be responsible [153]. Coincidentally, the 275th residue in the M-loop (β270–β286) of β3 tubulin also has the otherwise conserved serine residue in other β tubulin isoforms substituted to alanine. Interestingly, the M-loop and the
leucine cluster do not bind taxol directly although these two regions are proximal to the taxol binding pocket. As a result, mutations in these sites may weaken taxol binding. Indeed, β3 tubulin has been found to bind the least tritium-labelled taxol analog compared to other tubulin isoforms [153]. Thus, lower taxol affinity may contribute to the insensitivity of β3 tubulin to taxol. By contrast, earlier data using potentially less pure β tubulin isoforms obtained from immunoaffinity chromatography suggested the opposite, that β3 microtubules bind more taxol molecules compared to mixed brain tubulin [154].

1.7.1 Taxol and taxol resistance in cells and cancer patients

Taxol is thought to cause mitotic arrest in cells, via the spindle assembly checkpoint (SAC), eventually resulting in cell death. Knockdown of a SAC silencing protein enhances taxol cell-killing effects, making the drug effec-
tive at nanomolar concentrations [155]. Previously, taxol was thought to cause cell death by mitotic arrest of cells in metaphase, however, this view has been challenged as multipolar spindles were observed at clinically relevant taxol concentration [156, 157]. These dividing cells can then exit metaphase and separate into two or more daughter cells that eventually die as a result of chromosome missegregation, potentially due to loss of some essential genes [157].

In cancer patients, the dose and frequency of taxol administration for chemotherapy can vary from 1 to 4 weekly cycles, with dosage between 80 mg/m² and 175 mg/m², with slightly lower dose for weekly cycle and higher dose administered for three-week cycle treatment [157–160]. Early clinical studies determined taxol concentration in steady-state plasma to be about 0.85 ± 0.21 µM after taxol administration, from 48 refractory ovarian cancer patients [161]. More recent data from breast cancer patients suggest taxol plasma concentration to be about 2364 ng/mL (around 2.8 µM) [158]. However, plasma concentration does not necessarily resemble the situation in tumour tissue.

Data availability of intratumoral taxol concentration is limited due to the requirement for biopsy after taxol administration. On the contrary, taxol intracellular concentration in cell lines is better understood. In early studies, taxol was reported to accumulate to a micromolar level in HeLa cells treated with nanomolar concentration of taxol [162]. A more recent study reported similar intracellular taxol accumulation in MDA-MB-231 cells (breast cancer cell line) and primary breast tumours in patients [157].

Taxol needs to penetrate cell membranes to bind microtubules residing in cells. Thus, cells can acquire taxol resistance through up-regulation of P-glycoprotein to facilitate efflux of taxol, thereby reducing intracellular taxol concentration. Inhibition of this plasma membrane transporter can reverse taxol resistance in MCF-7/DX1 overexpressing P-glycoprotein [163]. In addition, overexpression of certain tubulin isoforms in particular cancers have been associated with taxol treatment response and cancer prognosis, as reviewed by Parker et al. [164]. For example, overexpression of β3 has been linked to poor prognosis in
ovarian cancer and breast cancer [148, 165]. High mRNA expression of class I β tubulin (tubulin encoded by gene *TUBB*) and β3 tubulin had correlations with docetaxel resistance in breast cancer patients wherein the mRNA overexpression was then proposed to be suggestive of treatment response [148]. Nevertheless, protein expression is always a dynamic process in cells, where downregulation of one isoform could potentially be compensated by overexpression of another, adding further complexity to tubulin-drug interactions. Interestingly, despite the correlation of overexpression of β3 tubulin and poor taxol response in uterine serous carcinoma patients, epothilones, which have overlapping binding site as taxol, seem to remain effective in vitro [166].

Other than resistance to taxol conferred by altered tubulin isoform expression, cells can acquire taxol resistance through other mechanisms such as PTMs. Acetylation and tyrosination of α tubulin have been associated with taxol resistance in lung cancer and breast cancer, respectively [167, 168]. IC 50 (50% inhibitory concentration) of patient-derived primary lung cancer cells were found to be higher corresponding to higher expression of acetylated tubulin as well as upregulation of acetylated tubulin observed in taxol treated mice xenografted with non-small cell lung cancer [167].

To clarify the picture, there is a need to isolate single isoform tubulin to study the effects of taxol and other MTAs on pure single isoform microtubules. Potentially, the responses of defined-isoform microtubules to drugs at defined concentrations might be useful to design better treatment regimes and predict treatment outcomes for cancer patients.

### 1.7.2 Confounding relationship between taxol and tubulin isoforms in different cancers

Overexpression of β3 tubulin has been linked to taxol resistance in breast cancer cell lines MCF-7 and MDA-MB-231 [165]. In contrast, some cell lines not only can acquire resistance to taxol after repetitive exposure, but can
even become dependent on taxol to maintain normal growth as are A549 non-small cell lung cancer cells [169]. In the development of this taxol resistant cell line, after A549 cells had been exposed to stepwise increasing taxol concentration, a few β tubulin isoforms were found to have increased RNA expression as determined by RT-PCR [149]. Clinically, overexpression of β3 tubulin is also associated with poor survival and treatment outcomes in non-small cell lung cancer and ovarian cancer patients [170–172].

There are data to suggest that overexpression of β3 tubulin predicts better prognosis and taxane treatment response in ovarian clear cell adenocarcinoma [173]. In contrast, another study showed a correlation of taxol resistance with increased β3 tubulin expression in ovarian serous adenocarcinoma [170]. In addition, increased expression of tubulin isoforms other than β3 have also been associated with taxol resistance. Class I, II and IVa β tubulins were elevated in taxol-resistant tumour-bearing ascites compared to untreated primary tumour samples in ovarian cancer patients [149]. Interestingly, and in apparent contradiction, another study suggested that overexpression of β tubulin class I, II or IVb did not confer taxol resistance, whereas mutation of class I β tubulin was necessary to confer resistance to taxol [174].

Protein expression is always a complex process and increased mRNA expression does not always translate into an increase in protein production. For example, expression of β tubulin is governed by self-regulatory mechanisms whereby an increase in soluble β tubulin in cells causes mRNA to be co-translationally degraded [175]. These seemingly contradictory findings again suggest taxol resistance is multifactorial in cells and emphasise the necessity of in vitro experiments using specific single isoform tubulins.

1.7.3 Taxol associates with other microtubule effectors

Despite being a type of MSA, when used at low concentration, taxol has also been reported to promote microtubule catastrophe in the presence of
EB3 [176]. Other than EB3, taxol effects can also depend on other effectors. Tau promotes cooperative taxol binding depending on tau concentration [177]. Strikingly, using taxol, an MSA, together with an MDA, can produce synergy rather than counteraction. When used together at low concentration, taxol and vinorelbine (a type of MDA) were shown to have synergistic effects against human melanoma cell lines [178]. Interestingly, the order of how taxol and vinblastine (another MDA) were added, sequentially or simultaneously, has been shown to be relevant for the synergistic or antagonistic effects [179].

1.7.4 Taxol can recognise and modulate microtubule conformation

Although taxol binds microtubules stoichiometrically, taxol and some other MSAs at substoichiometric concentrations were found to accumulate at microtubule defects that result from a local mismatch of protofilament numbers. These defective sites can also serve as stable rescue hotspots that allow microtubules to undergo multiple catastrophe events and rescues at the same spot [180]. In addition, these authors have previously shown that fluorescent taxol derivatives have more prolonged and frequent accumulation at minus ends [181], raising a question about structural recognition of microtubule by taxol. Other than recognition of microtubule defects, early study also showed a lower dissociation constant $K_d$ of fluorescent taxol derivative from GMPCPP microtubules, which suggests preferential binding of taxol molecules to certain lattices [182].

The microtubule-stabilising effects of taxol are believed to result from blocking of lattice compaction upon GTP hydrolysis, as GDP-taxol microtubules were found to adopt an expanded lattice compared to GDP microtubules without taxol [183]. Intriguingly, the microtubule lattice appears to respond to taxol differently depending on when taxol is added during microtubule preparation with slightly shorter dimer rise of $81.8\,\text{Å}$ reported when taxol was supplied post-microtubule assembly and $82.3\,\text{Å}$ for microtubules with taxol added during microtubule assembly, in comparison to GDP microtubules which have dimer rise
of 81.5 Å [30, 184].

Although higher microtubule stability often correlates to an expanded lattice, which could be part of taxol’s stabilising mechanism, another MSA, peloruside, does not cause lattice expansion, giving dimer rise of 81.0 Å [184]. Thus, microtubule stabilisation is not necessarily accompanied by lattice expansion.

Besides driving correlated stabilisation and lattice expansion, taxol may also mediate lateral interaction of protofilaments as taxol focuses protofilament number and causes a more elliptical microtubule cross-section compared to drug-free microtubules [184]. Since taxol added to pre-formed microtubules does not cause expansion and yet microtubules still can be stabilised, the stabilising mechanism of taxol could be due to strengthened lateral interactions between tubulin dimers. This was demonstrated by a 1.7 Å displacement of the fragments of the M-loop and the S9-S10 loop of β tubulin together with GTP hydrolysis-induced microtubule compaction for microtubules copolymerised with doublecortin with taxol added post-assembly [115]. Taxol stabilisation was then proposed to be due to the locking of the M-loop conformation, effectively blocking protofilaments from peeling outwards [115].

Interestingly, different parts of taxol molecules are responsible for different activities. Although taxol binds minimally to free tubulin, baccatin III (taxol precursor, corresponding to the core structure of taxol without the C13 side chain) binds curved free tubulin. Strikingly, despite the absence of microtubule-stabilising effect, baccatin III-bound microtubules adopted an expanded lattice like taxol-stabilised microtubules do [185]. Furthermore, taxol was found to bind the straight conformation of free tubulin but not the curved state in this work. X-ray diffraction patterns suggest that microtubules adopt expanded lattice regardless of when taxol was added during microtubule preparation or afterwards [185], in contrast to what has been proposed based on cryo-EM [184] and for doublecortin bound microtubules with taxol added post-microtubule assembly [115]. Nevertheless, the experimental data suggest different modules within taxol
molecules are responsible for microtubule binding, expansion and stabilising effects.

Allosteric effects of taxol have been demonstrated using chicken erythrocyte tubulin, where several regions of tubulin, including sites in α tubulin, were found to undergo conformational changes upon taxol binding. Xiao and colleagues employed hydrogen to deuterium exchange (HDX) coupled with mass spectrometry to assess potential structural changes caused by taxol. In this assay, amide protons, which are accessible to solvent, can be exchanged to deuterium, except for proline due to its lack of exchangeable amide hydrogen atoms. HDX of GDP-tubulin dimers and of microtubules assembled in the presence of GTP with or without taxol were compared [186]. Certain sites such as regions in α tubulin and parts of the H1-S2 and H2-S3 loops in β tubulin had less hydrogen exchanged into deuterium in GDP-taxol microtubules, potentially because these sites are buried and thereby protected from exposure to deuterium due to conformational changes induced by taxol binding [186]. This suggests allosteric effects of taxol binding to microtubules since these sites are distal to the taxol binding site. As a result, divergent residues in these sites could also potentially modulate microtubule conformations triggered by taxol binding. The allosteric effects of taxol and modulations of divergent residues in particular regions might begin to explain why GDP-taxol α1b/β3 microtubules have mainly 12 protofilaments whereas the GDP-taxol α1b/β2b microtubules have roughly similar distribution across 12- and 15-protofilament arrangement as these two β tubulins have a few residues differ in the H1-S2 loop [3].

1.8 Microtubule and kinesin-1 interactions

Motor proteins participate in different essential cell activities. Different motors bind different cytoskeletal filaments such as myosin binds actin whereas kinesin and dynein bind microtubules. As the name suggests, these proteins move around cells to allow intracellular transport or sliding of filaments by transform-
ing chemical energy obtained from nucleotide hydrolysis, usually breakdown of ATP, to mechanical energy.

Polarity of microtubules allow kinesins and dyneins walk towards the plus and minus ends, respectively, for anterograde and retrograde transport in neurons. While some kinesins are mainly responsible for intracellular transport, others facilitate cell division by binding antiparallel microtubules and slide them apart. Although most kinesin families walk towards the plus end, some kinesins are minus end directed or can sometimes track microtubules bidirectionally.

Kinesin-1, also known as conventional kinesin, is perhaps one of the most well-studied kinesins. Like most kinesin family members, kinesin-1 is plus-end directed and is responsible for anterograde transport of intracellular materials in axons, where most of the microtubules have the plus end pointing towards the axon tip and the minus ends pointing towards the cell body. In human, there are three kinesin-1 isoforms: KIF5A, KIF5B and KIF5C.

Kinesin-1 is a heterotetramer formed by two heavy chains and two light chains. Although the light chains play some roles in cargo binding, they are not required for the transport of mitochondria and some synaptic precursor vesicles. The heavy chains consist of several main structures, motor domains at the N-terminal, a neck linker which joins the neck with motor domain and a tail domain connecting to the neck through a hinge. In order to prevent futile ATP hydrolysis and traffic congestion along microtubules with kinesin-1 not transporting cargoes, kinesin-1 is autoinhibited by the cargo-binding domain with the coiled-coil domain folding at the hinge and binding to the motor domain [187]. Kinesin-1 microtubule tracking is activated upon cargo binding to the tail of the heavy chain and/or light chain [188].

Kinesin-1 walks along microtubules in a hand-over-hand manner via neck linker docking following hydrolysis of the bound ATP. Kinesin binds microtubules within single tubulin heterodimer, bridging the intradimer interface. Kinesin-microtubule binding mainly involves loop 2, loop 11 and helix 6 of kinesin with α tubulin; together with helix 4 and helix 5 of kinesin at the intradimer
interface of tubulin; and loop 8 of kinesin near the plus end of β tubulin [117].

Kinesin-1 is a processive molecule which has a stall force of about 6–8 pN, a run length of 1 µm and walking velocity of about 0.8 µm/s along microtubules. The binding affinity of kinesin to microtubules is nucleotide-dependent, being strongest in the absence of nucleotide (in the rigor state), slightly less strong in the ATP and ADP.Pi states and weak-bound in its ADP state. The stabilisation of microtubules conferred by kinesin-1 corresponds to the binding affinity of kinesin, with the most stabilisation by rigor kinesin, followed by AMPPNP (slowly-hydrolysable ATP analog) kinesin-1, and almost no stabilisation in ADP state [116]. In addition to microtubule stabilisation, kinesin-1 can expand the lattice of GDP mixed-isoform brain microtubules [116, 117]. Other data suggest a slight compaction of GMPCPP microtubules upon kinesin-1 binding [31].

Other than causing conformational changes, kinesin-1 has been shown to preferentially bind GMPCPP microtubules [117, 189] and acetylated microtubules [106]. However, some kinesins are not yet known to recognise different microtubule lattice conformation. The preferential binding of kinesin-1 to microtubules has been shown to be dependent on loop 11 of kinesin which can recognise the nucleotide state and/or conformation of microtubules [117, 190]. The increased microtubule-activated ATPase activity on GMPCPP microtubules was also abolished for a KIF5C mutant with the loop 11 swapped to that of KIF1A [189]. The in vivo relevance remains unclear – kinesin-1 does not preferentially bind GTP-rich microtubules when cargo-bearing native kinesin-1 is used [191].

Interestingly, microtubule nucleotide state can regulate kinesin-1 running velocity whereby kinesin runs 36% faster along GMPCPP microtubules than GDP-taxol microtubules, corresponding to 32% faster microtubule activated ATPase rate [189]. This raises a question about how kinesin can recognise less than 3% of dimer rise difference between GMPCPP and GDP microtubules, 83.95 Å and 81.76 Å, respectively [31].
1.9 Single isoform tubulin engineering – what has been achieved so far?

Conventionally, mammalian brains, which contain a complex mixture of tubulin isoforms, are used as a source of tubulin for laboratory studies. The yields are often in the range from 100 to 300 mg of tubulin per 1 kg of pig brain [192]. Brain tubulin purification is achieved by cycles of tubulin polymerisation-depolymerisation which could also potentially bias towards certain tubulin isoforms.

The commonly used bacterial expression system is unsuitable for tubulin expression due to the absence of chaperones and other co-factors which are essential for proper tubulin folding [193]. It is possible to obtain single isoform tubulin from yeast, but yeast is unable to fold human tubulins (Cross lab, unpublished). Instead, the baculovirus expression system has been widely used for single isoform mammalian tubulin expression in the last decade [194]. The recombinant tubulins can dimerise with insect cell tubulins, so double-tagging schemes have to be employed to ensure faithful isolation of all-recombinant tubulin heterodimers in high purity. Several different tagging schemes have been used, such as an internal histidine tag in α tubulin, N-terminal tag in α tubulin and cleavable tags, with different strategies in β tubulin, see Table 1.1. Yields are reported to be between 1 and 5 mg/L of culture depending on the tagging schemes and cell lines used [2, 3, 53, 194].

1.9.1 Microtubule dynamics and structures can be dictated by tubulin isoforms

Thanks to insect cell expression, there is increasingly emerging evidence suggesting a relationship between tubulin isoform and the regulation of metazoan microtubule dynamics and structure. Minoura and colleagues first demonstrated the use of baculovirus-insect cell expression system for single iso-
Table 1.1: Selected single isoform tubulin and mutants with different tagging schemes expressed using baculovirus expression system.

<table>
<thead>
<tr>
<th>Isoforms</th>
<th>Tagging Schemes (α tubulin/ β tubulin)</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human α1b/β3</td>
<td>C-terminal 8x histidine tag /C-terminal FLAG tag</td>
<td>[194]</td>
</tr>
<tr>
<td>Human α1a/β3</td>
<td>internal histidine tag in acetylation loop /C-terminal cleavable FLAG tag</td>
<td>[2]</td>
</tr>
<tr>
<td>Human α1b/βI+βIVb</td>
<td>native no tags, isolated from human embryonic cell line</td>
<td>[130]</td>
</tr>
<tr>
<td>Human α1b/β2b</td>
<td>cleavable N-terminal 10x histidine tag</td>
<td>[3]</td>
</tr>
<tr>
<td>Human α1b/β3</td>
<td>/cleavable C-terminal Strep tag</td>
<td></td>
</tr>
<tr>
<td>Human α1b-E254A/β3 mutant</td>
<td>internal 6x histidine tag in acetylation loop /cleavable C-terminal Strep tag</td>
<td>[62]</td>
</tr>
<tr>
<td>Human α1b-E254D/β3 mutant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drosophila α1-K40R/β1</td>
<td>cleavable C-terminal 8x histidine tag /cleavable C-terminal FLAG tag</td>
<td>[53]</td>
</tr>
<tr>
<td>Drosophila α1-K40R/β1-Y222</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human α1b-E254N/β3 mutant</td>
<td>internal 6x histidine tag in acetylation loop /cleavable C-terminal Strep tag</td>
<td>[4]</td>
</tr>
</tbody>
</table>
form tubulin. They found that C-terminally tagged K40R (α tubulin mutant, incapable of acetylation) human α1b/β3 microtubules glided slower than pig brain microtubules on kinesin-coated surfaces, whereas the similarly tagged yeast microtubules travelled 5% faster than native yeast microtubules, suggesting the underlying role of PTMs and/or intrinsic structural differences between tubulin isoforms in governing microtubule-kinesin motility [194].

Several studies have since been performed to elucidate the properties of single isoform microtubules. Ti and coworkers studied the effects of tubulin isoforms on microtubule structure and stability. They showed that β tubulin isoform composition tunes microtubule protofilament numbers and microtubule depolymerisation rates by revealing intermediate protofilament numbers and intermediate depolymerisation rates of microtubules assembled from mixture of two single isoform α1b/β2b and α1b/β3 tubulins [3]. Further, GMPCPP α1b/β2b microtubules have predominantly 14 protofilaments unlike the majority of GMPCPP α1b/β3 microtubules which adopt a 13-protofilament arrangement. In contrast, Vemu et al. found that GMPCPP α1a/β3 microtubules have 14 protofilaments instead of 13 for GMPCPP α1b/β3 as suggested by Ti et al. [2, 3]. The discrepancy might be due to the different tagging schemes used. Although it seems less likely, the 2-residue difference in the α tubulins might also cause this structural distinction.

In addition to microtubule structure, Vemu et al. also showed that growth rate and catastrophe frequency of microtubules can be regulated by varying the ratio of α1a/β3 and α1b/βI+βIVb tubulins (here the β1 refers to class I tubulin encoded by TUBB gene, not the tubulin encoded by TUBB1 gene which is mainly expressed in platelets) [130]. They also demonstrated separately that α1a/β3 microtubules depolymerised slower than α1b/βI+βIVb microtubules [2, 130]. In contrast, Ti et al. found that α1b/β3 microtubules depolymerised faster than α1b/β2b [3]. Strikingly, βI and βIVb tubulins share higher sequence similarity with β2b tubulin than β3 tubulin. Thus, one might expect that both α1b/βI+βIVb and α1b/β2b microtubules should exhibit similar dynamics when compared to α1a/β3 microtubules. Again, the tagging schemes used might be
relevant as the $\alpha_{1a}/\beta_3$ tubulin had internal his-tag in $\alpha$ tubulin, whereas the $\alpha_{1b}/\beta_{I+}\beta_{IVb}$ tubulin was native tubulin purified from a human embryonic kidney cell line (tsA201).

Due to the high sequence divergence of CTTs, one might argue that CTTs are mainly responsible for encoding the different dynamics and structural properties of single isoform microtubules. However, CTTs of $\beta$ tubulins seem to be less involved in microtubule dynamics. Pamula and workers have demonstrated that microtubules prepared from chimeric $\beta$ tubulins, by swapping over $\beta_3$ and $\beta_{2b}$ tubulin cores and tails, had catastrophe frequencies similar to the corresponding core of full-length constructs, albeit the $\alpha$ tubulin was mixture of insect ones and human $\alpha_{1a}$ tubulin [195]. On the other hand, different $\alpha$ tubulin isoforms, which mainly differ in the CTTs, can mediate microtubule dynamics [196]. The seemingly different capability of CTTs of $\alpha$ and $\beta$ tubulins to regulate microtubule dynamics might potentially be due to the CTT locations. Although CTTs of both tubulins are not resolved by cryo-EM to date, the CTTs of $\alpha$ tubulins directly connect to the C-termini of H12 helices which lie near the interface of interdimer interaction site of two longitudinally adjoining tubulin dimers, see Figure 1.2(b) for locations of C-termini of H12 helices of both $\alpha$ and $\beta$ tubulins. Combining all these pieces of evidence, CTTs of $\alpha$ tubulins may indeed participate in microtubule dynamic regulations where the CTTs of $\beta$ tubulins may modulate microtubule interactors such as kinesin and dynein.

Interestingly, microtubules of the same isoform with even just two different single residue mutagenesis at the same position may generate distinct microtubule structures. The $\alpha_{1b}/\beta_3$ microtubule mutants $\alpha$-E254A and $\alpha$-E254N, which are GTP hydrolysis-deficient, adopt negative and positive lattice twists, respectively [4]. In addition, the $\alpha$-E254N microtubule mutant adopts two distinct lattice configurations that have different binding affinities for EB [4]. Thus, seemingly similar “loss of function” (incapable of GTP hydrolysis) point mutations result in distinct lattice structures, emphasising the high plasticity of microtubules.
Single isoform tubulin expression using baculovirus-insect cell system has not only opened the door for the studies of the roles of individual tubulin isoforms but also for the investigations of molecular mechanisms and pathways, for example, a Y222F β tubulin mutant has been used for the study of microtubule nucleation [53].

In this work, single isoform α and β tubulins of human or zebrafish were co-expressed in insect cells and purified to elucidate pure human α1b/β3 and zebrafish α1c/β4b microtubule properties; in particular, their response to taxol and interaction with kinesin-1. Hopefully, this will allow us to gain more understanding of tubulin resistance mechanism to taxol and provide a basis for microtubule drug development specific to different tubulin isoforms.
Chapter 2

Materials and Methods

2.1 Reagents

All reagents were obtained from Sigma-Aldrich unless otherwise stated.

2.2 Sf9 cell maintenance

Sf9 cells were grown in ExCell 420 media, supplemented with 100 U/mL penicillin and 100 U/mL streptomycin, as suspension cultures with shaking at 120 rpm at 28°C. The culture was split to a density of $0.5 \times 10^6$ cells/mL when it reached about $2.0 \times 10^6$ cells/mL. For transfection, cells were grown as adherent cultures and incubated at 28°C without shaking to obtain P1 recombinant baculovirus whereas suspension culture was used for propagation of P2 and P3 virus, see Section 2.4 for virus preparation.
2.3 Cloning and Bacmid Generation

The \( \alpha \) tubulin (human TUBA1B; NP_006073 and zebrafish tuba1c; NP_001098596) and \( \beta \) tubulin (human TUBB3; NP_006077 and zebrafish tubb4b; NP_942104) genes were obtained from GeneArt with codon optimisation for expression in Sf9 cells. A 21-base pair long L21 leader sequence AACTCC-TAAAAACCGCCACC was placed at the 5’ end of tubulin genes to enhance protein expression. A 5-amino acid linker GGSGG was positioned at the 3’ end of tubulin sequences, followed by the C-terminal tags, 8x histidine and FLAG tags for \( \alpha \) and \( \beta \) tubulins, respectively, both of which were then cloned into the pBIG1 plasmid backbone, see Figure 2.1.

Respective \( \alpha \) and \( \beta \) tubulin genes were assembled into pBIG1 plasmid as described by [197]. Briefly, the individual \( \alpha \) and \( \beta \) tubulin genes were amplified by PCR with linkers flanked both ends to allow subsequent Gibson Assembly to integrate into pLIB vector backbone. The products were then propagated in Escherichia coli (E. coli) DH5\( \alpha \), positive clones were selected by antibiotics, followed by plasmid extraction using MiniPrep kits (Qiagen). Gene expression cassettes of individual \( \alpha \) and \( \beta \) tubulins were then amplified by PCR using primers flanked the region between promoter and terminator sequences of individual pLIB plasmids encoding respective \( \alpha \) and \( \beta \) tubulins. The resulting amplicons were joined together by Gibson Assembly with pBIG1 as vector backbone to generate pBIG1-\( \alpha \)-\( \beta \) tubulin constructs which encoded both \( \alpha \) and \( \beta \) tubulins. The Gibson Assembly products were again propagated in E.coli and positive clones were selected using appropriate antibiotics. The resulting pBIG1 plasmids had dual pH promoters (polyhedrin promoters) each upstream of \( \alpha \) and \( \beta \) genes, with Tn7R and Tn7L transposition sites flanked the polygene cassettes, to allow integration into the baculovirus genome. The tubulin-encoding genes of the plasmids were verified by sequencing.

The pBIG1 plasmid encoding both \( \alpha \) and \( \beta \) tubulins was then integrated into baculovirus genome through Tn7 transposition by transformation of DH10EmBacY E.coli carrying the viral genome. The positive clones were
selected through blue-white screening, in addition to kanamycin, gentamicin, tetracycline antibiotic selection. The recombinant baculovirus DNA (bacmid DNA) was extracted using MidiPrep kits, with overnight DNA precipitation in isopropanol at $-20^\circ$C. After a wash in 70% ethanol, the air-dried pelleted DNA was resuspended in water. The successful insertion of tubulin sequences in bacmid was verified through PCR using M13 forward and reverse primers. Bacmid DNA was kept at 4°C for storage, and used within a week after preparation.
2.4 Sf9 cell transfection and virus propagation

Transfection for generation of P1 virus was performed using adherent Sf9 cultures. Sf9 cells were seeded onto a 35 mm petri dish with 2 mL of culture with a density of $0.5 \times 10^6$ cells/mL, followed by 30 minutes of incubation to allow cell attachment to the surface. Meanwhile, transfection mix was prepared by mixing 200 µL of ExCell 420 media with 3 µg of bacmid DNA. After mixing well, 6 µL of FuGeneHD (Promega) was added. The transfection mix was allowed to stand for 15 minutes before dripping the entire volume onto the culture. P1 virus was usually harvested 4 to 5 days after transfection, after which time almost the entire cell population typically expressed YFP. The virus was harvested by removal of cell debris by centrifugation at 750 x g for 5 minutes. The tube of virus-containing supernatant was wrapped with aluminium foil and kept at 4 °C for storage, and normally used within two weeks of preparation, maximally one month.

Although viral titre was not determined, P1 virus has been reported to have a generally lower titer and is relatively less practical to produce at a larger scale, therefore higher titre P2 or P3 virus was used to infect cells for recombinant tubulin expression instead. P2 virus was generated by adding 1 mL of P1 virus in every 100 mL of Sf9 culture at a density of about $1.0 \times 10^6$ cells/mL, followed by shaking incubation for 3 days. P3 virus was propagated using P2 virus in this same procedure. Generally, the use of virus with higher propagation number is not recommended as more defective interfering particles can accumulate over time, eventually impacting protein expression. Both P2 and P3 virus were harvested by spinning down the culture at 750 x g for 5 minutes and the supernatant was filtered through 0.45 µm PVDF syringe filter. The virus was kept at 4 °C for storage and normally used within two months.
2.5 Main buffers and stock solutions

All buffers were reconstituted in ultrapure water (18.2 MΩ, Elga), unless otherwise stated.

KPEM buffer for tubulin purification and microtubule dynamics assay was constituted of 100 mM PIPES (piperazine-N-N’-bis(2-ethanesulphonic acid) (Melford, P40140), 2 mM EGTA (ethylene glycol-bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid) and 1 mM MgSO$_4$ with pH adjusted to 6.9 using KOH. BRB 80 buffer for microtubule gliding assay was composed of 80 mM PIPES, 1 mM EGTA and 1 mM MgCl$_2$.

Nucleotide stocks: 100 mM GTP was reconstituted in KPEM whereas 100 mM ATP was prepared in 100 mM PIPES supplemented with 100 mM MgSO$_4$. In both cases, pH was adjusted with KOH to 6.9 and 7.0, respectively. All solutions were filtered through a 0.2 µm filter and stored at $-80^\circ$C.

Due to low solubility of epothilone B and taxol, these reagents were reconstituted in DMSO at 10 mM separately, stored at $-20^\circ$C.

2.6 Tubulin expression and purification

Sf9 cultures were infected with 30 mL of P2 or P3 virus per litre of culture at a density of 2.0×10$^6$ cells/mL and incubated at 28 $^\circ$C for approximately 54 hours. The following purification steps were carried out at 4 $^\circ$C or on ice unless otherwise stated. Cells were collected by spinning down at 750 x g for 20 minutes and subsequently washed with ice-cold PBS, pelleted down again, followed by flash-freezing in liquid nitrogen. Frozen pellets were stored at $-80^\circ$C until use.

Tubulin was purified using AKTA fast protein liquid chromatography, except for FLAG purification which was performed manually. All purification
buffers were KPEM based and supplemented with 1 mM ATP and 1 mM GTP on purification day, unless otherwise stated. All buffers used were degassed with 0.2 µm filter on purification day. Cell pellets were thawed on ice and resuspended in equal volume of buffer KPEM supplemented with 25 mM imidazole, 250 mM KCl, 1 mM DTT and 0.05% CHAPS, 0.5 M 3-(1-pyridinio)-1-propane sulfonate, lysed using a dounce homogeniser with about 50 to 60 strokes. Whole cell lysate was clarified at 300,000 x g for 30 minutes. The supernatant was passed through a 5 mL HisTrap HP column (Cytiva) at rate of 2 mL/min, tubulin was eluted around 350–500 mM imidazole (isoform dependent) in KPEM, supplemented with 250 mM KCl. The eluates were pooled and diluted in an equal volume of KPEM without KCl. This reduced the final KCl concentration to 125 mM to facilitate better recombinant tubulin binding to anti-FLAG resin. The diluted sample was then rolled with 4 mL of anti-FLAG® M2 resin (A2220, Merck) for an hour. The slurry was flowed through a 10 mL column to allow resin packing, followed by elution with 5 sequential column volume of 250 µg/mL FLAG peptide in KPEM supplemented with 125 mM KCl.

The pooled FLAG eluates were mixed with three parts of KPEM without KCl to reduced KCl concentration to about 30 mM, to improve protein binding to a Capto HiRes Q 5/50 column (Cytivia), flowed at rate of 0.5 mL/min. Tubulin was eluted with KPEM supplemented with 400 mM KCl, 0.5 mM GTP and 0.5 mM ATP. The pooled eluates were buffer exchanged into KPEM without KCl and no nucleotide by passing over a HiPrep 26/10 desalting column (Cytiva) at 3 mL/min and finally concentrated to approximately 40 µM using an Amicon® centrifugal concentrator (Ultracel® regenerated cellulose membrane with a 30 kD molecular weight cut off). Tubulin was aliquoted and snap-frozen in liquid nitrogen. The aliquots were stored in a vapour phase liquid nitrogen tank and quickly thawed between hands before use.

The tubulin heterodimer concentration was determined by absorbance at wavelength 280 nm. The molar extinction coefficients used for α1b/β3 tubulin and α1c/β4b tubulin were 108,390 M$^{-1}$cm$^{-1}$ and 107,110 M$^{-1}$cm$^{-1}$, respectively.
2.7 Kinesin-1 expression and purification

The kinesin construct had a C-terminal 6x histidine tag linked via a thrombin cleavage site LVPRGS to full-length D. melanogaster kinesin heavy chain (gene Khc; NP_476590) in pPK113 vector backbone [198], see Figure 2.2.

![Diagram of Kinesin Construct](Created with SnapGene®)

**Figure 2.2:** Kinesin construct. The kinesin sequence is linked to a C-terminal 6x histidine tag via a thrombin cleavage site.

Kinesin was expressed in *E. coli* BL21 pLysS strain, with the culture maintained at 24°C, 180 rpm. As the protein expression was leaky, IPTG induction was omitted. This gave a better yield of soluble, full-length kinesin.
dimers in the lab. The culture was harvested when it reached an optical density of 0.9 at 600 nm wavelength. The following steps were carried out at 4°C unless otherwise stated. The culture was pelleted at 15,000 x g for 6 minutes before washing in ice-cold PBS and pelleted down again. The pellet was then resuspended in 3× pellet mass of buffer composed of 10 mM Tris-base, 4 mM Mg-acetate, 250 mM K-acetate, 1 mM TCEP, supplemented with 100 µM ATP, complete protease inhibitor (Roche), 0.5% vol/vol Triton X-100, >125 unit/mL Benzonase® Nuclease, 0.1 mg/ml lysozyme, with pH adjusted to 8.0 with acetic acid. The mixture was then lysed by sonication (Misonix, S-4000 Ultrasonicator) with 6 cycles of 10 second pulses at 35 Amps followed by 30-second cooling after each burst. The lysate was clarified at 20,000 x g for 20 minutes and the supernatant was filtered through glass wool.

The filtered supernatant was then applied to a 1 mL HiTrap Talon crude column (Cytivia) and eluted with around 100–150 mM imidazole in 10 mM Tris-base, 4 mM Mg-acetate, 250 mM K-acetate, supplemented with 1 mM TCEP and 100 µM ATP. The pooled affinity-purified eluates were then further purified by application to a 1 mL HiTrap Q HP column (Cytivia) followed by elution with 200 mM NaCl in 10 mM Tris-base, 4 mM Mg-acetate, 250 mM K-acetate, supplemented with 1 mM TCEP and 100 µM ATP. The pooled eluates were mixed with glycerol to 20% glycerol and snap-frozen in liquid nitrogen for storage.

2.8 Recombinant tubulin verification by western blotting

The samples were lysed using NuPAGE™ LDS sample buffer according to manufacturer’s protocol. The samples were then run on a gel (NuPAGE Bis-Tris, Invitrogen) in MOPS buffer for about an hour at 200 V. The bands were transferred onto a nitrocellulose membrane (IB301002, ThermoFisherScientific) using an iBlot gel transfer device for 7 minutes with the default P0 method. The membrane was blocked using 1× casein (B6429, Merck) for one hour at room temperature, followed by overnight incubation with primary antibodies in 1×
casein at 4°C. The concentrations of primary antibodies used for detection of α tubulin and β tubulin were 0.5 µg/mL anti-α tubulin antibody (ab7291, Abcam) and 1.0 µg/mL anti-β tubulin antibody (ab6046, Abcam), respectively, whereas the presence of tags, for confirmation of recombinant tubulin, was also verified, with the use of 1.0 µg/mL anti-6x histidine antibody (MA1-21315, Invitrogen) and 0.2 µg/mL anti-FLAG antibody (ab1162, Abcam), respectively.

The primary antibodies were subsequently washed with PBS supplemented with 0.01% tween 20 (PBS-T) for 3 times, 5 minutes each. The membrane was incubated with fluorophore-conjugated secondary antibodies (goat anti-mouse A32723 or goat anti-rabbit A11011, Invitrogen) in 1× casein for an hour at room temperature, washed again with PBS-T as above. The bands on gel were visualised and imaged with G:BOX (ChemiXRQ, SynGene).

2.9 Coverslip cleaning to produce a hydrophilic surface

Dark-field microscopy allows visualisation of unstained particles, hence, coverslips and slides used need to undergo rigorous cleaning to produce a good signal to noise ratio and also to condition the coverslip surface for subsequent microtubule attachment.

Coverslips and slides were sonicated for 30 minutes in ultrapure water (18.2 MΩ, Elga) brought to boil before addition of 3% Neutracon, then 3 rounds of 6-min sonication followed by 3 rinses each with water only, and 8 rounds of 5-min sonication with 1 rinse each in water only. Coverslips were then dipped in absolute ethanol and air-dried with compressed nitrogen gas. These dry coverslips were then wrapped in lens tissue and stored in a zip-loc bag. The glass slides and coverslips were then plasma-cleaned (HPT-200, Henniker Plasma) with air at power 50% for 5 minutes before use.
2.10 GMPCPP seed mix

Microtubules were grown off biotinylated GMPCPP-stabilised seeds for attachment to glass slides via a NeutrAvidin-biotin linkage. To make GMPCPP seed mix, 26 µM of porcine brain tubulin constituted of 1:1:8 ratio of biotinylated tubulin (Cytoskeleton) : HiLyte 488 tubulin (Cytoskeleton) : unlabelled tubulin (previously purified as described [199]), was mixed with 1 mM GMPCPP (Jena Bioscience) in KPEM, and incubated on ice for 15 minutes. The solution was clarified with AirFuge (A-110 fixed angle rotor, Beckman Coulter) for 5 minutes at 4 °C at 20 psi to remove large aggregates. The supernatant was snap-frozen in liquid nitrogen and stored in a liquid nitrogen tank. After thawing the seed mix, seeds were assembled at 37 °C for 30 minutes, diluted 20× in KPEM, pelleted at 20 psi with AirFuge for 5 minutes at room temperature, and finally resuspended in an equal volume of buffer. This seed solution was suitably diluted before use for optimal coverslip decoration.

2.11 Flow cell chamber preparation for microtubule dynamics assay

Flow cells were assembled from one microscope slide (≈ 1.0 mm thick, 76 mm × 26 mm) and one coverslip (thickness no. 1.5, 22 mm × 22 mm), so as to sandwich two stripes of double-sided tape (Scotch tape, 3M), about 5 mm apart, producing a channel of about 10 µL in volume. The channel was filled with 0.2 mg/mL poly(L-lysine)-PEG-biotin (SuSoS) in PBS for 15 minutes, unbound PLL-PEG-biotin was then washed out with KPEM. Subsequently, 45 µL of 1 mg/mL NeutrAvidin (Thermo Fisher Scientific) was introduced and incubated for 5 minutes, again washed out. Biotinylated microtubule seeds were then introduced and incubated for a few minutes to allow seed binding. Unbound seeds were finally washed out with 1% tween 20 in KPEM buffer.
2.12 Dark-field Microscopy

Dark-field images were acquired with an electron-multiplying charge-coupled device (EMCCD) camera (Andor, iXon DU 897) fitted to Nikon E800 microscope with a 100× objective (Plan Fluor NA 0.5–1.3 variable iris, Nikon). Samples were illuminated with a 100 W mercury short-arc lamp (102D, Ushio) connected to the microscope with a fibre optic light scrambler (Technical video) and an oil condenser with an N.A. of 1.43–1.20 (Nikon). Light was filtered through a cold mirror and green interference filter with 500–568 nm band pass (Nikon). Epifluorescence was achieved using a stabilised mercury lamp (X-cite exacte, Lumen Dynamics) with light pipe connection to the microscope. Fluorescent and dark-field imaging was achieved through a combination of a single-band bandpass filter for excitation (FF01-469/35-21.8-D, Semrock), through a dichroic mirror (Q495lp, Chroma) and a long pass filter for emission (ET500lp, Chroma). Electronic shutters were used to control the exposure time of sample to light and switching over between dark-field illumination and epifluorescence. Microscope temperature control was maintained at 30 °C using a custom-made chamber fitted with a heater (Air-Therm ATX, World Precision Instruments).

Microscope and camera were controlled by Metamorph software (Molecular Devices). For dynamics experiments, 300 ms and 200 ms exposure times were used for dark-field and epifluorescence imaging, respectively, with 1 frame of EGFP for every 300 frames of dark-field images, 1 frame per second and 160 nm per pixel.

2.13 Microtubule dynamics assay

Dynamics assays were performed with different concentrations of tubulin in KPEM buffer (pH 6.9, 100 mM PIPES, 2 mM EGTA and 1 mM MgSO4), supplemented with oxygen scavenging system (4.5 mg/mL glucose, 0.2 mg/mL
glucose oxidase, 35 µg/mL catalase and 0.5% (v/v) β-mercaptoethanol), 1 mM GTP, 1 mg/mL BSA and 0.2% (v/v) tween 20. All solutions were clarified by AirFuge at 4°C at 20 psi for 5 minutes to remove large particles before use. Tubulin solution was then introduced into flow cell channel. All microtubule dynamics assays were performed using GMPCPP pig brain seeds to allow microtubule extension.

To prevent evaporation, flow cell ends were sealed with vacuum grease after tubulin mix was introduced into seed-decorated flow cell channels. For buffer exchange, if needed, channel ends were left unsealed and covered with parafilm. In this case, the microscope chamber was pre-humidified with wet tissue to minimise evaporation loss.

2.14 Microtubule gliding assay

Sonicated-cleaned coverslips were coated with 1% nitrocellulose in amyl acetate before use. The coverslips were coated by placing a drop of nitrocellulose solution onto a lens tissue, wiping the damped lens tissue across the coverslip surface. Flow cell chamber was assembled as above, with two coverslips of different sizes instead (thickness no. 1.5), with one 50 mm × 22 mm and one 22 mm × 22 mm coverslips. The channel volume was about 5 µL. Microtubules for all gliding assays were assembled using single isoform tubulin (or pig brain tubulin for segmented microtubule preparation) mixed with 5% HiLyte 647 (or HiLyte 488 if another colour was needed to distinguish two different microtubules) labelled porcine brain tubulin (Cytoskeleton) for visualisation under fluorescence microscope.

GMPCPP pig brain or single isoform microtubules were assembled using 10 µM of tubulin, supplemented with 1 mM GMPCPP, followed by incubation at 37 °C for 30 minutes, then diluted 2× in KPEM buffer with or without 20 µM of taxol (final taxol concentration was 10 µM in this case) with a further 30-minute
incubation.

GDP-taxol pig brain or single isoform microtubules were prepared in several ways: 1) microtubules assembled in the presence of both taxol and GTP, 2) microtubules prepared in GTP with taxol added post-microtubule assembly and 3) microtubules assembled in taxol alone without GTP. For 1), tubulin was polymerised at 40µM tubulin in 1 mM GTP in KPEM, incubated for 30 minutes and taxol was added the same way as GMPCPP taxol microtubules with final taxol concentration of 10µM or 500nM, supplemented with 1 mM GTP. For 2), tubulin was polymerised in GTP as 1) but after 30 minutes of incubation, the reaction was diluted 20× in KPEM and supplemented with taxol to 10µM and with 1 mM GTP. For 3), 20µM tubulin was supplemented with 10µM taxol and incubated for an hour at 37°C.

Segmented microtubules were prepared by mixing individually prepared single isoform microtubules and/or pig brain microtubules as follow: respective microtubules, labelled with different fluorophores, were spun down separately at 20 psi using AirFuge for 5 minutes at room temperature to remove free tubulin, the pellets were gently rinsed then resuspended in one-half of the original volume composed of 1 mM GTP and 10µM taxol, different microtubule solutions were mixed together and incubated overnight at room temperature to allow spontaneous end-to-end joining.

The flow cell channel was decorated with kinesin as follow: the channel was filled with kinesin solution in 0.1mg/mL α-casein in BRB 80 buffer with 5 minutes of incubation at room temperature. Unbound kinesin was washed out with BRB 80 buffer supplemented with 1 mM DTT and 2 mM ATP. Suitably diluted GDP-taxol or GMPCPP-stabilised microtubules, usually about 40 – 50× dilution, in BRB 80 buffer supplemented with 2 mM ATP, 1 mM DTT, oxygen scavenging system (as above) with or without 10µM taxol. Microtubule gliding was observed under eduWOSM (Warwick Open Source Microscope for education) at room temperature about 22°C where images were acquired at 1 frame every 5 seconds. Briefly, the illumination was achieved using a LED light engine and im-
age acquired with EMCCD camera, 185 nm per pixel. For details of eduWOSM, please see https://wosmic.org/projects/eduwosm/Nick_eduWOSM_talk.php.

2.15 Image analysis

For dark-field microscopy, stage drift often happened after a prolonged period of imaging. Therefore, time series was first processed to correct for stage drift by using the Fiji plugin Manual Drift Correction.

Kymographs were generated by overlaying a 11-pixel straight line along microtubules using the Fiji plugin Multi Kymograph. Polymerisation and depolymerisation rates of microtubules were hand-traced by fitting a segmented line to the end(s) of each microtubule. Therefore, each segmented line is treated as one data point (n=1). For microtubule dynamics quantification, only the growth rates detected within the first 15 minutes of imaging were considered to avoid the effects of tubulin denaturation over time as microtubule growth rates are dependent on tubulin concentrations whereas depolymerisation rates of the entire 30 minutes were included. In addition, to minimise errors, microtubules with length changes which were shorter than 1 µm were not considered for analysis. The mean and median rates of microtubule growth and depolymerisation were obtained by dividing the sum of respective rates (represented by individual line segments) by the total number of segments.

Velocity and angular change of gliding microtubules were hand-traced by clicking on the ends of advancing microtubules using the Fiji plugin MTrackJ. For all gliding velocity plots, “n” represents “instantaneous” velocity of advancing microtubules between two consecutive frames for x number of randomly selected microtubules, except for the low taxol β4 microtubules and GDP-taxol segmented microtubules where n = 1 represents mean velocity of each individual gliding microtubules over tracking time.
2.16 Statistical analysis

Mann-Whitney U test was performed to compare microtubule dynamics and microtubule gliding velocities. Population distributions with two peaks, whenever applicable, were fitted to a double Gaussian distribution.
Chapter 3

Results

3.1 Tubulin expression and optimisation

In order to obtain single isoform tubulins with minimal contaminants, tags were inserted at the C-termini of both $\alpha$ and $\beta$ tubulins. Human $\alpha_{1b}/\beta_3$ tubulin, which has previously been successfully purified from insect cells, and $\alpha_{1c}/\beta_4$ tubulin of zebrafish, were selected for protein expression. The human and zebrafish tubulins will be referred to as $\beta_3$ and $\beta_4$ tubulin isoforms, respectively, from now on for convenience. The tags were placed at the C-termini to ensure full-length protein purification. The tubulin-encoding plasmids were not designed to have cleavable sites to keep the number of foreign amino acid residues to a minimum and to retain the possibility of using the tags for future fluorescent labelling.

Although I could not completely rule out the impact of tags, especially in terms of MAP binding due to the role of CTTs, microtubules prepared from these tubulin isoforms are capable of kinesin binding as demonstrated by microtubule gliding assay. Furthermore, despite the identical tagging strategy for both $\beta_3$ and $\beta_4$ microtubules, they exhibited dramatic difference in gliding velocities in
GDP-taxol state (0.431 ± 0.049 µm/s vs 0.672 ± 0.069 µm/s) but not GMPCPP state (both about 0.7 µm/s), see Section 3.4.1 and 3.4.5. In addition, previous work has demonstrated that yeast microtubules prepared from tubulin with identical tagging strategy glided only 5% faster than the wild type microtubules [194]. Thus, it is less likely that the tags are causing this drastic difference in GDP-taxol microtubule gliding velocities driven by kinesin.

P1 virus (the virus harvested after transfection of insect cells), is usually low in viral load, leading to inefficient protein expression. Therefore, P2 virus, which has higher viral load, was expanded from P1 virus by adding the P1 virus to fresh Sf9 cell culture in 1:100 ratio, followed by incubation to allow virus propagation for generation of more virus. P2 virus was used for tubulin expression for the first couple of tubulin preps whereas P3 virus was used subsequently due to virus shelf life. P1 virus shelf life is reported to be short, about 1 month, whilst P2 virus can be stored for up to 6 months. P3 virus was therefore employed for protein expression with the use of P2 virus as “stock” to further expand P3 virus whenever needed, to minimise the number of times required to perform Sf9 cell transfection for generation of P1 virus. It is not recommended to use higher passage number of virus for protein expression (higher than P4), as the viral particle can sometimes become defective.

Recombinant protein production increases over time after Sf9 cell infection, but less protein remains in the soluble fraction at late stage. Therefore, it is important to identify the optimal time to harvest the cells, the time at which the maximal concentration of protein is present in the soluble fraction. The baculovirus genome I used has YFP gene expression cassette integrated, driven by a pH promoter, which serves as a reporter for virus-infected insect cells. The YFP fluorescence intensity is also somewhat correlative to tubulin expression as both YFP and tubulin expression are driven by pH promoters upstream of them. The optimal culture harvest time for tubulin was determined from timecourse experiments where cultures were sampled regularly, samples run on a gel and YFP signal intensity determined by fluorescence spectrophotometry. Gel band intensities of tubulin present in the supernatant over time were then compared.
to those of the corresponding pellet fractions. The time when the most recombi-
nant tubulin remained in soluble fraction was determined to be around 53 hours
post-cell infection, see Figure 3.1(a) for a typical recombinant tubulin expression
timecourse in which soluble tubulin decreased at a late stage.

Hi5, Sf9 and Sf21 insect cells were tested to select for the cell line that
gives the best tubulin expression. The Hi5 cell line has been reported to give
higher protein expression in general. In my hands, with the same virus to culture
volume ratio used for Sf9 cells, the population of Hi5 cells expressing YFP under
microscope was only 10-20%, which potentially suggests low protein expression
(data not shown, tubulin expression was not quantified due to small population
of cells expressing YFP). My instinct is that more virus would be needed to create
a larger infected population. However, with the current amount of virus used
(30 mL of virus per 1 L of culture), it is not practical to generate an even larger
volume of virus due to sample handling. Another potential reason for the low
number of infected Hi5 cells could be the presence of heparin, which is needed for
culture adaptation. Hi5 cells are recommended to be revived as adherent cells
when received from vendors, and can subsequently be adapted to suspension
culture as desired. The adaptation to suspension culture requires the addition
of heparin to avoid cell aggregation, especially for the first few passages, prior to
heparin addition can be omitted. However, heparin has been reported to interfere
with baculovirus infection [200]. At the point of attempted protein expression,
heparin was still required, even though the amount required was relatively small
compared to the initial dose. In addition, although unlikely, I could not rule out
the possibility that despite only a 10-20% infected cell population, Hi5 cultures
might give a better yield overall than Sf9 cultures (in which almost all cells are
infected by virus), provided the protein expression efficiency is high. In short,
with more optimisation, Hi5 cells might possibly give the best tubulin yield. Sf21
cells were also tested but again the tubulin expression was not appreciably higher
than that of Sf9 cells.

Recombinant tubulin dimerises with endogenous insect cell tubulin. Therefore, in order to obtain pure recombinant tubulin free from host tubu-
Figure 3.1: Single isoform tubulin purification. (a): Recombinant tubulin in supernatant fractions of Sf9 cell lysates sampled at 0, 48, 66 and 72 hours post-infection. Bands were detected by western blot against histidine and FLAG tags. (b): Gel image showing tubulin fractions at different purification steps. 1: whole cell lysate; 2: NiNTA purified; 3: FLAG purified; 4: IEX purified. Image cropped and regrouped from two whole gels from one tubulin prep.

lin, a double-tagging scheme was employed where the first and second steps of purification select for the respective recombinant $\alpha$ and $\beta$ tubulins. After 2-step purification, there were still some contaminant proteins present. A third purification step was therefore added to remove contamination through ion exchange chromatography, see Figure 3.1(b). Initially, buffer exchange into KPEM without additional salt was done using a spin concentrator. However, the time taken to exchange buffer from high salt (about 400 mM KCl) to almost no salt (< 1 mM) is appreciable and could result in tubulin aggregation over time. Hence, buffer exchange was eventually achieved using a desalting column to allow rapid process.
Precipitation of protein was sometimes observed during different tubulin preps with the degree of precipitation being time-dependent despite purification was performed on ice or at 4°C and the concentrations of ATP and GTP used were the same for different tubulin isoforms and no nucleotide regeneration system was used. Due to the labile nature of free tubulin in solution and the long hours needed for a 3-step purification, the process was paused overnight at a stage at which tubulin was bound to the column, to avoid tubulin self-interaction. Although not quantified, β4 tubulin seems to give more precipitation than β3 tubulin.

Tags placed at the C-termini of both tubulins could potentially affect microtubule interaction with MAPs as CTTs are exposed on the microtubule surface. My dynamics experiments show that the additional tags do not severely impact microtubule dynamics whilst microtubule gliding assays suggest that microtubules slide unimpeded across kinesin-1 decorated surfaces. Although not compared with the tagless version of microtubules due to unavailability of materials, the fact that these two single isoform microtubules exhibit different properties implies that these distinctions may arise from divergent sequences in tubulin core rather than the CTTs.

3.2 Dynamic instability of single isoform microtubules

Microtubule dynamic instability depicts the properties of a microtubule undergoing stochastic phases of growth and shrinkage, termed rescue and catastrophe. While the growth rate of microtubules is tubulin concentration-dependent, the shrinkage rate is not greatly affected by free tubulin concentration [42]. However, a more recent view has suggested some involvement of free tubulin concentration in setting microtubule shrinking rates [43].

Previous work has demonstrated that single isoform microtubules have distinct dynamics [2, 3, 130]. However, these studies mainly focus on growth
rate and catastrophe frequency [130] or on depolymerisation of GDP-taxol microtubules and GMPCPP microtubules with or without MAPs [3]. Here, I mainly concentrated on how variations in tubulin isoform composition affect microtubule lattice stability, with depolymerisation rates as the readout. In addition, as the protofilament number of β3 or β4 microtubules (copolymerised with trace fluorescently labelled and biotinylated porcine tubulin) is unclear, all the GMPCPP seeds used here for microtubule nucleation were prepared from the mixture of unlabelled pig brain tubulin with fluorescently labelled pig brain tubulin and biotinylated pig brain tubulin in 8:1:1 ratio.

Several experimental conditions were optimised to ensure microtubules grow and shrink in a time frame suitable for imaging and analysis. Microtubule catastrophe frequency is inversely proportional to free tubulin concentration [42]. If tubulin concentration is too high, microtubules need to be observed over an unfeasibly long period of time for catastrophe to be observed. If concentration is too low, microtubules shrink while they are still short. Shrinkage following a catastrophe is typically rapid, albeit temperature and salt concentration-dependent. Due to instrumental limitation, the frame rate of my dark-field imaging cannot go over 1 frame per second. For all these reasons, short microtubules are not ideal for reliable quantitative measurements. However, long microtubules could also pose challenges as thermal fluctuations sometimes make microtubules go in and out of focus, preventing the capture of this entire rapid shrinkage process. As a result, tubulin concentration has to be optimised to allow microtubules to grow to desirable lengths.

In the beginning, experiments were conducted at 25°C as working close to room temperature makes sample handling easier. However, the yield of recombinant tubulin is relatively low compared to brain tubulin purification. By bringing temperature up, tubulin nucleation concentration is decreased, allowing the use of relatively less material. Considering these points, imaging temperature was raised to 30°C, midway between the optimal temperatures for zebrafish and human. I largely focused on microtubule plus ends here as the minus ends did not show much dynamicity under the conditions used here.
3.2.1 Microtubule growth rate and depolymerisation rate measurement

Kymographs were usually fitted using a segmented line to trace individual microtubule growth and shrinkage, see Figure 3.2. For depolymerisation events, a segmented line was fitted only in the cases where it was obvious that there were two phases or more, corresponding to approximately a 2-fold difference between rates. Otherwise a single phase fit was used. For growth events, when a single line connected between the point of the start of a microtubule growth and the point before catastrophe did not match the tip of the growing microtubule, a multi-segmented line was fitted.

3.2.2 Single isoform $\beta_4$ microtubules shrink slower than $\beta_3$ microtubules

Single isoform $\beta_3$ and $\beta_4$ microtubules show clearly distinct depolymerisation rates. $\beta_4$ microtubules depolymerised at $303.3 \pm 140.6$ nm/s, slower than $\beta_3$ microtubules, which depolymerised at $688.3 \pm 217.7$ nm/s (mean ± SD), see
Figure 3.3(a) red line, when compared using a single Gaussian fit. Rapid depolymerisation of β3 microtubules occurred in a single phase. Depolymerisation of β4 microtubules, by contrast, could occur either in a single phase or in two phases. The histogram of depolymerisation rates of β4 microtubules, together with kymographs, as seen in Figure 3.2, reveals more than one rate population with mean depolymerisation rates at 481.9 ± 193.2 nm/s and 274.3 ± 67.5 nm/s, when fitted with a double Gaussian distribution, see Figure 3.3(a) blue line for double Gaussian fit.

Since multiphasic depolymerisation events usually started with fast depolymerisation and converted to slower depolymerisation, I further analysed the data by defining three event classes, see Figure 3.3(b): firstly, depolymerisation events fitted with only one line without change of rate, termed “NoMid”; secondly, rates obtained from the first line segment, the initial depolymerisation rate, termed “IniWithMid”; and lastly, the remaining rates, “Mid”, obtained from the second line segment onward. These rate populations were then plotted against microtubule length, to see the relationship between depolymerisation rates and microtubule tip positions from the seeds. However, this shows that the “IniWithMid” population had a wide distribution spanning the slow and fast rates, without strong indication of correlation with microtubule tip position. Similarly, the “NoMid” population also had a rather broad rate distribution. In contrast, the “Mid” population appears to have more events occurred with slow rate.

3.2.3 Single isoform microtubules have relatively similar growth rates

In contrast to their markedly different depolymerisation rates, β3 and β4 microtubules grew at relatively similar rates at 19.1 ± 5.1 nm/s and 16.4 ± 5.2 nm/s (mean ± SD), respectively, when assembled at 12 µM tubulin, see Figure 3.4(a).

Tubulin association and dissociation rate constants can be obtained
Figure 3.3: Depolymerisation rates of single isoform microtubules. (a): Single isoform β3 microtubules depolymerise faster than β4 microtubules where n = 404 and 467, respectively. (b): β4 microtubules occasionally show distinct shrinking rates along the same microtubules. IniWithMid: initial rate of a microtubule depolymerisation event with change of rate; Mid: subsequent depolymerisation rates of a microtubule after the initial rate; NoMid: depolymerisation rate of a microtubule without change of rate. Data from 3 experiments. µ and σ (1 & 2): mean and standard deviation (on the left and right), a: area of peak on the left.
from the plot of microtubule growth rates as a function of tubulin concentrations [201], see Figure 3.4(b), with following equation:

\[ V_{\text{growth}} = k_{\text{on}} \times C - k_{\text{off}} \]  \\
(3.1)

where \( V_{\text{growth}} \) is microtubule growth rate (which can be directly obtained from experiment), \( k_{\text{on}} \) is association rate constant, \( C \) is tubulin concentration and \( k_{\text{off}} \) is dissociation rate constant.

The y-intercept of the plot denotes \( k_{\text{off}} \) whereas the gradient represents \( k_{\text{on}} \). These parameters can be obtained in the future with a few more concentrations tested to provide a more robust fitting (note: the growth rates of microtubules at concentrations other than 12 µM were obtained from only one experiment each).

### 3.2.4 Mosaic microtubule dynamics is dependent on the ratio of tubulin isoforms

In order to study how tubulin compositions tune microtubule dynamics, I prepared tubulin mixtures by varying the ratio of \( \beta_3 \) and \( \beta_4 \) tubulins while keeping the total tubulin concentration the same. I varied the overall concentration ratio in the assembly reaction; at this stage, I did not have fluorescently labelled tubulins, so cannot quantify the isoform ratio in the assembled polymers.

Depolymerisation rates of these mosaic microtubule (assuming random copolymerisation of different tubulin isoforms into microtubules) show a general trend, with the rate increasing as the \( \beta_3 \) tubulin content increased, see Figure 3.5. The response appears nonlinear. At 12 µM of total tubulin concentration, including 20% of \( \beta_4 \) tubulin in the reaction mix for microtubule assembly did not detectably change the depolymerisation rate. These mosaic microtubules showed a very similar depolymerisation rate at 754.2 ± 354.7 nm/s (median ±
Figure 3.4: Growth rates of single isoform microtubules. (a): $\beta_3$ microtubules and $\beta_4$ microtubules grow at $19.1 \pm 5.1 \text{ nm/s}$ and $16.4 \pm 5.2 \text{ nm/s}$ (mean $\pm$ SD) at 12 $\mu$M tubulin, respectively, where $n = 493$ and 551. (b): The growth rates of two single isoform microtubules as a function of tubulin concentrations are relatively similar at 10, 12, 15 and 18 $\mu$M tubulin where $n = 52, 493, 161$ for $\beta_3$ microtubules at 10, 12 and 18 $\mu$M tubulin; and $n = 119, 551, 117$ for $\beta_4$ microtubules at 10, 12, 15 $\mu$M. Data were obtained from 3 experiments for 12 $\mu$M set whereas only 1 experiment was performed for other concentrations. $\mu$: mean, $\sigma$: standard deviation. The grey shaded area of regression line represents confidence interval.
SD) to that of pure β3 microtubules at 746.7 ± 324.6 nm/s. Even an equimolar mixture of β3 and β4 tubulins did not slow the shrinking rate much relative to pure β3 microtubules, with rate at 724.7 ± 287.2 nm/s.

### 3.3 Microtubule dynamics in taxol

Previous reports have suggested lower taxol sensitivity of β3 tubulin. To test this point, I compared β3 and β4 microtubule dynamics in the presence of taxol.

To my surprise, when single isoform microtubules were assembled in the presence of taxol from GMPCPP pig brain seeds, they showed distinct structural response. At sufficiently high taxol concentration, 500 nM, kinks formed in β4 microtubules. These were absent when 100 nM taxol was used. β3 microtubules did not generate distinct kinks even with 1 µM and 5 µM of taxol used (data not shown). β4 kinks relaxed upon taxol washout, see Section 3.3.3.

#### 3.3.1 Microtubule stabilisation in different taxol concentrations

The minimal taxol concentration required to stabilise microtubules was isoform dependent. At 250 nM taxol, catastrophes were still occasionally observed along with rescues for β3 microtubules whereas most β4 microtubules resisted catastrophe at 50 nM taxol in the presence of free tubulin during 30 minutes of observation, see Figure 3.6(a).

Since β4 microtubules did not depolymerise within 30 minutes when assembled at 50 nM of taxol, free tubulin was washed out to trigger depolymerisation. Depolymerisation rates of taxol-stabilised microtubules were then obtained after washing out free tubulin with taxol solution at the concentrations at which the respective microtubules were assembled. When assembled at 50 nM
Figure 3.5: Growth and depolymerisation rates of respective mosaic microtubules. Microtubules were assembled at 12 μM of total tubulin concentration with increasing fractions of β4 tubulin starting from 0, 20, 50, 80 and 100%. Depolymerisation rates (top; median ± SD): 746.7±324.6 nm/s, 754.2±354.7 nm/s, 724.7±287.2 nm/s, 453.3±322.0 nm/s, 353.7±277.5 nm/s, n = 404, 198, 47, 186 and 467, respectively. Growth rates (bottom; median ± SD): 19.4±5.3 nm/s, 18.3±5.0 nm/s, 21.3±5.8 nm/s, 19.3±4.7 nm/s and 16.8±5.0 nm/s, n = 493, 240, 59, 210 and 551. The 0 and 100% data sets for both depolymerisation rates and growth rates were from Section 3.2.2 and 3.2.3. ns p ≥ 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001 and ****p < 0.0001, Mann–Whitney U test. The 20% and 80% data sets were from 2 experiments and the 50% data set was from 1 experiment only. The tails of the violins were trimmed.
taxol, β4 microtubules depolymerised at a rate almost half of that in the absence of taxol, at 152.7 ± 88.1 nm/s (median ± SD) compared to 303.3 ± 140.6 nm/s (mean rate of single Gaussian fit). Depolymerisation rates of β4 microtubules and β3 microtubules were about 6-fold slower than their counterparts without taxol, at respective 50.0 ± 36.1 nm/s and 108.4 ± 76.3 nm/s at 100 nM taxol, compared to 303.3 ± 140.6 nm/s and 688.3 ± 217.7 nm/s in the absence of taxol, see Figure 3.6(b) for taxol-stabilised microtubules and 3.3(a) for dynamics microtubules without taxol.

### 3.3.2 Local expansion of single isoform microtubule lattice

Microtubules composed of β4 tubulin developed kinks when a sufficiently high concentration of taxol (500 nM) was used during assembly. However, when 100 nM taxol was used, no kinks were observed for β4 microtubules. Interestingly, no obvious kinks developed in β3 microtubules for all taxol concentrations tested, see Figure 3.7.

### 3.3.3 Kinks can be relaxed by washing out taxol

The kinks produced during β4 microtubule assembly in the presence of 500 nM of taxol can be relaxed by washing in free tubulin without taxol and/or lower concentrations of taxol solution, suggesting taxol dissociation is relaxing the kinks, see Supplementary Movie S.1. Sometimes a few microtubules remained “curved” as they got stuck when crossing over. This was confirmed by the sudden “snap” back of microtubules upon depolymerisation beyond the crossover point.
Figure 3.6: Microtubule depolymerisation at different taxol concentrations. (a): Kymographs of β3 microtubules showing rescues (yellow arrow) following slower depolymerisation events (when compared to cases without taxol) when assembled at 12μM tubulin in the presence of 250 nM taxol and free tubulin whereas β4 microtubules grow processively even at 50 nM taxol. (b): Depolymerisation rates of microtubules in the absence of free tubulin where the rates of β4 and β3 microtubules are slowed down to 50.0 ± 36.1 nm/s and 108.4 ± 76.3 nm/s, where n = 313 and 89, respectively, at 100 nM taxol; and β4 microtubules at 50 nM taxol depolymerised at rate of 152.7 ± 88.1 nm/s (median ± SD) where n = 172. Data were from 3 experiments for β4 microtubules at both taxol concentrations whereas 2 experiments were performed for β3 microtubules.
3.3.4 The plus and minus ends of β3 microtubules are sometimes almost indistinguishable when assembled in 500 nM taxol

In most cases when tubulin was assembled in the absence of taxol, the minus end of microtubules of both isoforms were not dynamic. If the minus ends did grow, they shrank before they got long enough for robust quantification. When β3 tubulin was assembled in 500 nM taxol from GMPCPP seeds, plus and minus ends of some microtubules were difficult to distinguish as the minus end growth became processive, see Figure 3.8(a). The growth rates of both plus and minus ends of β3 microtubules did not show drastic difference regardless of the presence of 500 nM taxol which further suggests taxol suppresses catastrophe by slowing down shrinkage, so causing processive growth, see Figure 3.8(b). Note that the β4 microtubules were not analysed due to difficulty in tracking dynamics because of kinks.

Figure 3.7: Kinks of β4 microtubules induced by taxol. Microtubules of different isoforms have distinct structures when assembled in 500 nM of taxol solution with (a) β3 microtubules being relatively straight and (b) β4 microtubules kinky. Some of the β4 microtubule ends are “fuzzy” as they are not in the focal plane due to the kinks.
Figure 3.8: Microtubule plus and minus ends are indistinguishable sometimes in the presence of taxol. (a): Kymographs from dark-field imaging 30-minute time series showing β3 microtubule dynamics at 500 nM taxol (top), of which the polarity is almost indistinguishable whereas distinction can be easily made at 50 nM taxol (bottom). The edges of microtubules are outlined with yellow stroke. The β4 microtubules are not shown as they became kinky at 500 nM taxol. (b): Each grey line connects mean growth rates of plus and minus ends (represented by dots in blue and yellow, respectively) of individual microtubules with or without 500 nM taxol within first 15 minutes of imaging. Taxol allows processive growth of the minus ends but does not affect microtubule growth rate drastically. Note that the growth rates of minus ends which were not determined due to short length were deemed as 0 nm/s for illustration purpose despite their minimal growth. The growth rates (median ± SD) of plus ends are $15.6 ± 7.2$ nm/s and $19.4 ± 5.3$ nm/s, $n = 20$ and 109, whereas the minus ends are $7.8 ± 3.6$ nm/s and $8.0 ± 3.3$ nm/s, $n = 20$ and 19, with and without taxol, respectively, where the “not determined” minus ends were not considered as 1 n. Data set of no taxol was from Section 3.2.2 whereas the cases of taxol were from 2 experiments.
3.3.5 Segmented microtubules depolymerise in two distinct phases in the presence of taxol

Segmented microtubules consisted of sections of pure β3 and β4 tubulins were prepared by growing β4 microtubules from β3 microtubules extended from GMPCPP pig brain seeds. After the first segments of microtubules, which composed β3 tubulin, have grown sufficiently long, β4 tubulin solution was introduced to wash out remaining free β3 tubulin, followed by incubation to allow β4 microtubule extension from the “β3 seeds”.

The entire growth events of the exact segmented microtubules used for analysis were recorded to allow the identification of the β3-β4 junctions to ensure microtubules were indeed made up of two different tubulin segments. The spatial-temporal information on these junctions can be pinpointed from kymographs as few frames were disturbed during the flow of β4 tubulin, the time during which the flow ended was marked which can then be used to match the point when microtubules depolymerised into two different phases.

These microtubules depolymerised with two distinct rates corresponding to the distinct response of each isoform to taxol, see Figure 3.9(a). The experiments were initially performed in the absence of taxol and some microtubules did show two phases of depolymerisation, albeit the difference was less drastic than in the presence of taxol. The assembly of segmented isoform microtubules is tricky to perform without taxol for several reasons. Firstly, some microtubules with the first isoform grown directly off the seeds depolymerised and regrew before introduction of the second tubulin isoform, making the first segment too short for reliable quantification. Second, a population of microtubules with an already-formed first segment did not survive or survived only briefly after flowing in the second tubulin isoform, leading to a population of microtubules having only the second segment. Thirdly, microtubules could also depolymerise before the second isoform extension became long enough for robust quantification. Last but most importantly, microtubules with the first segment depolymerised while the second tubulin isoform was being incorporated into other microtubules would

\[ \text{...} \]
result in the release of the first isoform into the free tubulin pool (which ideally should contain only tubulin of the second isoform). Performing the experiment in low dose taxol (100 nM) prevents all these situations.

For segmented microtubules assembled in the presence of 100 nM taxol, only microtubules with an initial segment of β3 tubulin, grown off GMPCPP pig brain seeds, with a second segment of β4 tubulin were prepared, to avoid the complication that pure β4 microtubules extended directly from GMPCPP brain microtubule seeds can themselves sometimes depolymerise in two phases. These segmented microtubules depolymerised slowly in their β4 region and then converted to a fast rate as depolymerisation of the β3 segment began, at rates of $26.1 \pm 21.8 \text{ nm/s}$ and $210.7 \pm 142.3 \text{ nm/s}$ (mean ± SD), respectively.

Thus, 100 nM taxol amplifies the intrinsic difference in lattice stability between β3 and β4 microtubules from slightly over 2× in the absence of taxol to nearly 8× when taxol was present. The extent of suppression of depolymerisation rates by 100 nM taxol in these segmented microtubules differed slightly from that in the corresponding pure single isoform microtubules, see Section 3.3.1. In segments of segmented microtubules, depolymerisation proceeded at about 1/3 and 1/10 of that for β3 and β4 microtubules in the absence of taxol.

### 3.4 Microtubule gliding assay

In order to study microtubule-kinesin interactions, microtubule gliding assays were performed using full-length *D. melanogaster* kinesin-1, coated on to glass coverslips. Due to technical limitations of chemically labelling of recombinant tubulin, commercially available fluorescently labelled pig brain tubulin was employed to visualise microtubules, used at 5% of total tubulin concentration for all microtubule assembly.

Although β3 tubulin is more neuronal specific, pig brain microtubule
Figure 3.9: GDP-taxol β3-β4 segmented microtubules show two distinct depolymerisation rates. (a): Kymographs from dark-field imaging of microtubules composed of two segments of single isoform tubulin β3 and β4, extended from GMPCPP seeds (yellow bar) showing distinct depolymerisation phases at 100 nM taxol (left) whereas the difference is less obvious in the absence of taxol (right) within the same time scale (white bar represents 60 seconds). (b): Two distinct depolymerisation rates of microtubules in 100 nM taxol fitted to a double Gaussian distribution, n = 222. μ and σ (1 & 2): mean and standard deviation (on the left and right), a: area of peak on the left. Data from 3 experiments.
property is potentially more similar to that of β4 microtubule under certain conditions due to relatively low abundance of more divergent β3 tubulin compared to combined all other tubulin isoforms, which share more similar sequences to each other, in brain. This was later supported by the experimental results, in terms of microtubule gliding over kinesin-1 surface, see Section 3.4.1 and Section 3.4.6 for GDP-taxol single isoform microtubule and GDP-taxol pig brain microtubule gliding velocities. Herein, microtubules assembled from 5% fluorescently labelled microtubules are referred to as “pure” isoforms for convenience, to distinguish from mosaic microtubules. The assay was performed at a saturating Mg-ATP concentration of 2 mM, unless otherwise stated, to ensure ATP binding is not the limiting factor for microtubule gliding velocity.

3.4.1 GDP-taxol microtubules glide at different rates depending on tubulin isoform and taxol concentration

When 10 µM of taxol was used for microtubule assembly and gliding assays, β4 microtubules glided significantly faster (p < 0.0001, Mann-Whitney U test) at 0.672 ± 0.069 µm/s (median ± SD) than β3 microtubules with velocity of 0.431 ± 0.049 µm/s, see Figure 3.10(a), which is slightly more than a 50% increase for β4 over β3 microtubules. Despite the use of pig brain fluorescently labelled tubulin at 5%, this profound difference was still observed. Pig brain microtubules had a gliding velocity of about 0.650 µm/s, see Figure 3.14(a). Thus, gliding velocities of β3 microtubules and β4 microtubules could be slightly overestimated and underestimated, respectively. Nevertheless, this suggests that there are underlying factors that make microtubules of different isoforms glide at markedly different speeds in the presence of 10 µM taxol.
3.4.2 Gliding velocity of β4 microtubules can be tuned by adjusting taxol concentration

Due to this substantial difference in gliding velocity, and the known fact that β3 tubulin in pig brain microtubules has lower affinity for a taxol derivative, 2-(m-azidobenzoyl) taxol \[153\], I wondered if β4 microtubules would glide slower at lower taxol concentrations. Indeed, when 500 nM of taxol was used for β4 microtubules assembly as well as for gliding assay, the gliding rate of β4 microtubules decreased to 0.426 ± 0.064 µm/s, see Figure 3.10(b). The gliding velocity of β3 microtubules was not measured at this taxol concentration as microtubules depolymerised too rapidly to allow tracking. In fact, β4 microtubules also depolymerised slowly while gliding so the microtubules would appear to move even “slower” as the minus ends are advancing across kinesin surface and shrinking at the same time. This depolymerisation rate was accounted for in my quantification of gliding velocity in 500 nM of taxol. The lengths of individual microtubules were measured at the start and end of tracked sequences to obtain overall depolymerisation rates for the entire microtubules, including plus and minus ends, and this depolymerisation rate was added to the “apparent” velocity measurements, ie. for apparent gliding velocity measured at 0.400 µm/s, the 0.050 µm/s shrinking rate obtained was used to compensate for the “slowdown”, so the adjusted velocity was 0.450 µm/s. This adjusted velocity is slightly overestimated, as the shrinking rate is the total of both plus and minus ends.

Gliding velocity in this work was measured by tracking the advancing microtubule minus ends. In the future, microtubules can be labelled more sparsely and observed using TIRF, allowing the “landmarks” in the shaft to be used for more reliable tracking, especially for measurement of slowly shrinking microtubules and for automated tracking.
Figure 3.10: Gliding velocities of different GDP-taxol single isoform microtubules. (a) GDP-taxol single isoform β3 and β4 microtubules glide at significantly different velocities (p < 0.0001, Mann-Whitney U test) at 10 μM taxol at 0.431 ± 0.049 μm/s and 0.672 ± 0.069 μm/s (median ± SD), respectively, where n = 505 and 950, from respective 2 and 3 experiments, whereas (b) at 500 nM taxol, GDP-taxol β4 microtubules travel slower at a rate of 0.426 ± 0.064 μm/s, n = 59 microtubules, from 3 experiments.
3.4.3 Gliding velocity of mosaic microtubules can be tuned by adjusting the tubulin isoform ratio

Since GDP-taxol microtubules built of different tubulin isoforms glided at distinct rates at 10 µM taxol, it possible that tubulin isoform ratio would modulate microtubule gliding velocity.

Indeed, microtubules assembled from mixtures of two tubulin isoforms glided at rates between those of pure β3 and pure β4 microtubules, depending on the ratios of tubulin isoforms used, see Figure 3.11. Notably, mosaic microtubules assembled at a 1:1 β3:β4 tubulin molar ratio had a broader rate distribution which might represent two populations (fitted to a double Gaussian distribution) of gliding velocities at 0.441 ± 0.042 µm/s and 0.567 ± 0.054 µm/s (mean ± SD). Due to unavailability of fluorescent tubulins, I do not rule out tubulin isoforms of the same tend to cluster together to form microtubules with different compositions. However, mosaic microtubules show more different gliding patterns compared to single isoform microtubules, see Section 3.4.7, implying changes in material properties. This supports the idea that these two tubulin isoforms can indeed copolymerise to form mosaic microtubules to give rise to distinct behaviours different from the “raw materials”, although whether one isoform gets incorporated into microtubule better than another remains elusive.

The dependence of microtubule sliding rate on tubulin isoform ratio is non-linear. Mixing 20% β4 tubulin with 80% β3 tubulin gave a small but statistically significant decrease in gliding velocity, from 0.431 ± 0.049 µm/s for β3 tubulin alone to 0.424 ± 0.055 µm/s (median ± SD) (Mann-Whitney U test, p < 0.01), albeit only about a 1% reduction. By contrast, assembling mosaic microtubules from 80% β4 tubulin and 20% β3 tubulin gave an approximately 10% decrease in gliding velocity from 0.672 ± 0.069 µm/s for pure single isoform β4 microtubules to 0.609 ± 0.081 µm/s. Clearly one cannot simply infer microtubule properties, such as dynamics and interactions with MAPs, based on the ratio of tubulin isoforms used for microtubule assembly, not least because tubulin incorporation efficiency is not yet clear.
Figure 3.11: Gliding velocities of GDP-taxol mosaic microtubules are dependent on tubulin isoform compositions used for microtubule assembly. Microtubules assembled from higher ratio of $\beta_4:\beta_3$ tubulins generally glide faster (top). Double Gaussian fit of gliding velocity of mosaic microtubules prepared from equimolar of $\beta_3$ and $\beta_4$ tubulins (bottom left). Mixing in 20% of $\beta_4$ tubulin for microtubule assembly does not change gliding velocity of $\beta_3$ microtubules markedly, as suggested by the almost overlapping cumulative density functions of pure $\beta_3$ and 20% $\beta_4$ groups (bottom right). The pure $\beta_3$ and $\beta_4$ data are the same as Figure 3.10(a) for comparison. The gliding velocities of respective microtubules prepared from 0% to 100% of $\beta_4$ are as follow: $0.431 \pm 0.049 \mu m/s$; $0.424 \pm 0.055 \mu m/s$; $0.476 \pm 0.082 \mu m/s$; $0.609 \pm 0.081 \mu m/s$; $0.672 \pm 0.069 \mu m/s$ (median $\pm$ SD), $n = 505, 854, 845, 796, 950$, respectively. ** $p < 0.01$ and ****$p < 0.0001$, Mann–Whitney U test, from 3 independent experiments except for pure $\beta_3$ microtubules from 2 independent experiments.
3.4.4 Individual microtubules travel with relatively constant speed

Gliding velocity of individual microtubules was also analysed to rule out the possibility that populations of different gliding speeds, see Figure 3.11 middle panel 50% β4 mix, reflected individual microtubules switching their gliding speed during the course of imaging. Indeed, individual microtubules glided at relatively constant speed, from frame to frame, for the entire imaging time, see Figure 3.12 which also shows the change of travel direction of individual microtubules as they glided.

3.4.5 GMPCPP microtubules gliding velocity does not depend on isoform

Since GMPCPP pig brain microtubules have been previously reported to glide about 30% faster than GDP-taxol microtubules [189, 202], I wondered whether this could be true for my single isoform microtubules.

Interestingly but unexpectedly, despite the velocity difference being statistically significant, both GMPCPP β3 and β4 microtubules glided at about 0.7 µm/s, see Figure 3.13(a). This slight difference between isoforms stands in marked contrast to the huge difference in sliding rates I observed for the GDP-taxol lattices of these two isoforms. For GMPCPP β4 microtubules, the gliding velocity of $0.694 \pm 0.069$ µm/s is more similar to that of GDP-taxol microtubules at 10 µM taxol at $0.672 \pm 0.069$ µm/s which represents less than 5% increase. For β3 isoform, the GMPCPP microtubule gliding velocity of $0.705 \pm 0.044$ µm/s represents more than a 50% increase over the GDP-taxol rate of $0.431 \pm 0.049$ µm/s, see Figure 3.10.

Since β4 microtubules glided at different rates at different taxol concentrations, I wondered whether taxol would make GMPCPP β4 microtubule glide even faster. However, GMPCPP microtubules of both isoforms glided at similar rates with or without taxol, albeit the difference is statistically significant.
Figure 3.12: Instantaneous velocities of respective individual microtubules. Each individual point represents the velocity (x-axis) between two consecutive timepoints and the change in angle of gliding trajectory (represented by colour gradient) of individual microtubules (y-axis) between two consecutive timepoints. Data from Section 3.4.3.
(p < 0.0001, Mann-Whitney U test). For reasons as yet unclear, β4 microtubules in the presence of taxol exhibited enigmatic behaviour. To my surprise, GMPCPP-taxol β4 microtubules occasionally became stuck onto coverslips or travelled slowly. Some of these stuck microtubules managed to escape and when they did, they then moved with normal GMPCPP microtubule speed, see Figure 3.13(b). This was observed consistently in different experiments, although the proportions of stuck microtubules varied from experiment to experiment.

3.4.6 Preliminary data suggests that a GTP-like lattice state allows fast-gliding of GDP-taxol β4 but not β3 microtubules

Combining the results of single isoform GMPCPP microtubules and GDP-taxol microtubules suggests that kinesin might sense the expanded microtubule lattice characteristic of a “GTP/GTP-like” state. If so, my data suggest that in their GMPCPP states, both β3 and β4 microtubules have an expanded lattice, whilst in their GDP-taxol states, β3 lattices are compacted and do not expand upon taxol binding, whereas β4 lattices do.

To begin to test this hypothesis, I worked on GDP-taxol brain microtubules. These have been reported to have different dimer rises depending on whether taxol is added during or after microtubule assembly [184]. For this purpose, preliminary experiments were performed using pig brain microtubules prepared in different ways to allow direct comparison with literature. Pig brain tubulin was also polymerised in taxol without adding GTP, in addition to microtubules assembled in the presence of GTP with taxol added post-microtubule assembly. Indeed, brain microtubules prepared in these different ways did show distinct gliding velocities, see Figure 3.14.

The gliding velocity of pig brain microtubules assembled in the presence of both taxol and GTP was 0.645 ± 0.069 µm/s (mean ± SD, from 3 independent experiments, fitted to a single Gaussian distribution), see Figure 3.14(a). The gliding microtubules appear to fall into two populations (fitted to a double Gaus-
Figure 3.13: GMPCPP single isoform β3 and β4 microtubules have similar gliding velocities. (a): Both GMPCPP single isoform β3 and β4 microtubules travel at relatively similar speeds with or without taxol. The gliding velocities of GMPCPP microtubules of β3 without taxol, β3 with taxol, β4 without taxol and β4 with taxol are as follow: 0.705±0.044 μm/s, 0.718±0.046 μm/s, 0.694±0.069 μm/s, 0.666±0.202 μm/s (median ± SD), n = 690, 679, 653, 848 respectively. **** p < 0.0001, Mann-Whitney U test. Data from 3 experiments. (b): A population of GMPCPP-taxol β4 microtubules which are stuck initially sometimes manage to escape and keep on gliding as normal (red arrows), selected trajectories, not representative of the stuck population. Each track represents an individual microtubule.
sian distribution) with the minor, slower one peaking at 0.479 ± 0.023 µm/s and a faster one, making up 95% of the population, peaking at 0.646 ± 0.065 µm/s.

In contrast, taxol added post-microtubule assembly produced a majority of slow-gliding microtubules which glided at mean velocity of about 0.456 ± 0.060 µm/s, see Figure 3.14(b). A smaller 33% population of fast-gliding microtubules was also observed with mean velocity at 0.634 ± 0.063 µm/s potentially corresponding to the microtubules prepared in the presence of both GTP and taxol.

Caution is necessary as the presence of the minor population could be due to sample handling. In this case, tubulin was diluted 20× in 10 µM taxol to a concentration at which the free tubulin available was minimal so no new microtubules or relatively small population of microtubules would form upon mixing with taxol. However, it is difficult to ensure indeed no new microtubules were formed after taxol addition. Nevertheless, this preliminary result is highly suggestive. Experimental conditions will be optimised in the future to minimise potential heterogeneities. It may be possible, by varying the dilution factors, to see shifting of populations.

When pig brain microtubules were prepared in 10 µM of taxol alone in the absence of GTP, these microtubules also glided slower at approximately 0.453 ± 0.050 µm/s, see Figure 3.14(c) (preliminary data, from one experiment). This slow rate corresponds to that of the larger population of microtubules with 10 µM taxol added post-microtubule assembly, see Figure 3.14(b).

Previous literature reported a 30% sliding velocity difference between GMPCPP and GDP-taxol brain microtubules [189, 202]. Such a big difference was not observed in my experiments, rather, only about a 10% increase in velocity was observed for GMPCPP pig brain microtubules over GDP-taxol microtubules, see Figure 3.14(d).

I have shown that in addition, microtubule gliding velocity on kinesin-coated surface can vary depending on how microtubules are prepared.
Figure 3.14: Gliding velocities of GDP-taxol pig brain microtubules are dependent on when taxol is added during microtubule preparations. (a-c): GDP-taxol pig brain microtubules prepared in different ways. (d): GMPCPP pig brain microtubules.

(a): Gliding velocity of microtubules assembled in taxol shows a double Gaussian distribution with a smaller peak around $0.479 \pm 0.023 \mu m/s$ and a larger peak at $0.646 \pm 0.065 \mu m/s$; (b): Gliding velocity of microtubules with taxol added post-assembly shows a major peak around $0.456 \pm 0.060 \mu m/s$ and a smaller peak near $0.634 \pm 0.063 \mu m/s$; (c): Microtubules assembled in taxol only in the absence of GTP have gliding velocity peaks at $0.453 \pm 0.050 \mu m/s$. (d): GMPCPP pig brain microtubules have mean velocity at $0.709 \pm 0.060 \mu m/s$. $\mu$ and $\sigma$ (1 & 2): mean and standard deviation (on the left and right), $a$: area of peak on the left. Data from 3, 2, 1 and 3 independent microtubule preparations where $n = 447, 279, 137$ and 476 respectively.
3.4.6.1 Taxane-site binding drug epothilone B has similar effects to taxol

Since GDP-taxol microtubule gliding velocity is isoform dependent and the stage when taxol is added during microtubule assembly also matters for taxol-sensitive tubulin, I wondered whether other drugs whose binding sites overlap with that of taxol would have similar properties. I therefore tested another widely used MSA, epothilone B, also known as patupilone. The epothilone B-stabilised microtubules were prepared in the same way as taxol-stabilised microtubules, tubulin in GTP was incubated at 37 °C to allow nucleation. This reaction mix was then diluted with epothilone B solution with different dilution factors, followed by another half an hour of incubation. The reaction with lower dilution factor had more free tubulin available so that more newly-generated microtubules should form in the presence of epothilone B. In other words, with high enough dilution factor where free tubulin available is below critical concentration, there would be barely any microtubule assembly driven by epothilone B. Microtubules prepared in this way allowed us to have reactions with different populations of microtubules assembled in epothilone B.

In epothilone B experiments, β3 microtubules still glided slower than β4 microtubules when the reactions were prepared with 2× dilution factor, see Figure 3.15. However, β4 microtubules showed a broad spread of gliding velocity, which might suggest heterogenous microtubule populations. For example, this might reflect some microtubules formed before addition of epothilone B, and some after. In addition, gliding velocity of β3 microtubules in epothilone B remained the same no matter how they were prepared. β4 microtubules in epothilone B behaved as in the taxol experiments, producing distinct behaviour depending on whether the drug was added during or after microtubule assembly.

These preliminary data suggest that as for taxol-stabilised microtubules, epothilone B-stabilised microtubules have gliding velocities that depend on their tubulin isoform composition and on the stage at which the drug is added for microtubule preparation again matters.
Figure 3.15: Gliding velocities of GDP-epothilone B single isoform β3 and β4 microtubules are different and can vary with microtubule preparations. GDP-epothilone B β3 and β4 microtubules have distinct gliding rates at 0.392 ± 0.037 µm/s and 0.542 ± 0.103 µm/s (median ± SD), respectively, when assembled in the presence of epothilone B, see “2× dil”. Preliminary data from 1 experiment, n = 288, 273, 141, 264, 137 and 126, from top to bottom, respectively.
3.4.7 GDP-taxol single isoform microtubules have gliding patterns that are distinct from mosaic microtubules

GDP-taxol brain microtubules typically bend when gliding on a kinesin surface. At any one moment, some microtubules in the population move in a sinuous, snake-like trajectory. Sometimes microtubules move in circles. By contrast, GMPCPP microtubules travel in “straight” lines almost all of the time, see Figure 3.16(a).

Surprisingly, most single isoform GDP-taxol microtubules had a rather straight trajectory with a relatively large radius of curvature, with sinuosity or circling rarely observed, see Figure 3.16(b) and Supplementary Movie S.3 and S.4. However, when $\beta_3$ and $\beta_4$ tubulins were mixed together to form mosaic microtubules, the resulting microtubules from the mix of two single isoform tubulins behaved more like the mixed-isoform brain microtubules, see Supplementary Movie S.5. I quantified the variations in trajectory by determining the change of angle of a moving microtubule. Any one angle, $\theta$, is defined as the angle formed by the tip of a gliding microtubule between two consecutive times, $t_n$ and $t_{n+1}$, with respect to the x-y axis of the movie frames. If a microtubule travels in a “straight” trajectory, the angular change, $\Delta \theta$, should be close to zero ($\theta_1-\theta_2$), whereas if microtubules move sinusoidally or in circles, the angular change would be greater ($\theta_a-\theta_b$), see Figure 3.16(c) left panel. The empirical cumulative density function of the absolute value of angular change was plotted to compare microtubules prepared from different tubulin compositions, see Figure 3.16(c) right panel. Indeed, microtubules prepared from the 1:1 $\beta_3:\beta_4$ tubulin mixture had a larger population of microtubules with greater angular changes $\Delta \theta$. In addition, the change in gliding trajectory does not seem to be greatly biased towards clockwise or anti-clockwise direction at any one time, as suggested by Figure 3.16(d). Note, however, that this analysis was limited by some microtubules which formed loops with small radius of curvature or overlapping loops due to difficulty of tip tracking.
Figure 3.16: Gliding patterns of different GDP-taxol mosaic microtubules. (a): GDP-taxol pig brain microtubules sometimes loop around or glide with snake-like trajectory whereas GMPCPP microtubules have rather “straight” gliding trajectory (from left to right); (b): Microtubules gliding pattern can be tuned by tubulin isoform composition, with microtubules assembled from β3 tubulin only, 1:1 β3:β4 tubulins, β4 tubulin only (from left to right); (c): The angular change, Δθ, of gliding microtubules is the difference between the angles, θ, each of which formed by a gliding microtubule between two consecutive timepoints (left), and the cumulative plot of Δθ of different mosaic microtubules, pig brain microtubules (PB_MT) were included for comparison (right). Data from Section 3.4.3.
Figure 3.16: Gliding patterns of different GDP-taxol mosaic microtubules, continued. (d): Median angular change of gliding microtubules is more or less about 0° for all different microtubule preparations.
GMPCPP microtubules have smaller change in travel directions. The change of travel direction of GMPCPP microtubules is generally less dramatic compared to GDP-taxol mosaic microtubules and GDP-taxol single isoform microtubules. Angular change of gliding GDP-taxol pig brain microtubules is also shown here for comparison. n = 447, 505, 950, 476, 690 and 653 for GDP-taxol PB, GDP-taxol β3, GDP-taxol β4, GMPCPP PB, GMPCPP β3 and GMPCPP β4 microtubules, respectively. Data from Section 3.4.3, Section 3.4.5 and Section 3.4.6.

3.4.8 GMPCPP single isoform microtubules change travel direction only slightly

GMPCPP microtubules generally had rather straight gliding trajectory compared to GDP-taxol microtubules as exemplified by GMPCPP pig brain and β3 single isoform microtubules, see Figure 3.17. However, GMPCPP β4 microtubules did follow slightly less “straight” trajectories, compared to the GDP-taxol counterpart, both of which are still straighter than the GDP-taxol mosaic microtubules.
3.4.9 Segmented microtubules move along complex trajectories

I have found that β3 and β4 GDP-taxol microtubules travelled at different speeds on kinesin surfaces and that mosaic copolymers travelled at intermediate speeds. What happens to structured copolymers containing separate segments of my two isoforms? One possibility is that segmented microtubules could buckle or break at the junctions between segments. One might expect that when the leading segment of microtubule travels faster than the trailing segment, the microtubule might break. Whereas when the trailing end travels faster than the leading segment, the microtubule might buckle.

In order to make segmented microtubules, pre-assembled single isoform GDP-taxol and/or pig brain microtubules, composed of porcine brain tubulin labelled with different fluorophores, were pelleted down individually and the supernatant was removed. The pellets were gently resuspended and mixed together in buffer with 10 µM taxol and GTP and incubated overnight. The next day, segmented microtubules of different types, with β3 segment leading, or pig brain segment leading, or multi-segmented microtubules, were generated by spontaneous end-to-end joining. These glided differently depending on whether the slow β3 segment leads or trails the faster β4 segment or the pig brain segment.

To my surprise however, segmented microtubules composed of pig brain and β3 microtubule segments did not buckle or break easily. Instead, because the trailing pig brain tubulin segment moved faster intrinsically but was slowed down by the leading β3 segment, the trailing segment was forced to squiggle up into a series of mobile loops. Interestingly, when equimolar of ADP (2 mM) was mixed in, these squiggles became larger and more exaggerated, see Figure 3.18(a) and Supplementary Movie S.2.

Segmented microtubules made of only pure β3 and β4 segments behaved similarly, with trailing β4 segments squiggling up behind leading β3 segments. In contrast, when the fast-moving β4 segments were leading the β3 segments, the entire microtubules usually glided with rather “straight” trajectory, without
dramatic change in gliding direction, just like most of the GDP-taxol single isoform microtubules. However, I was unable to make many of these, so this type of segmented microtubules was not analysed in detail. The reason that relatively few β3-β4 segmented microtubules were formed may be that their ends are less compatible compared to the ends of the pig brain microtubules.

In addition, I found that the sliding speeds of the GDP-taxol segmented microtubules composed of pig brain tubulin and β3 tubulin segments were intermediate between the speeds of pig brain only microtubules and β3 only microtubules in GDP-taxol state, see Figure 3.18(b) for gliding velocity in 2 mM ATP plus 2 mM ADP. The extent of slowdown of segmented microtubules was generally proportionate to the length of leading slower-gliding β3 segments; Figure 3.18(b) inset shows the gliding speed difference between the set of all segmented microtubules with a leading β3 segment (regardless of length) and the subset with a short β3 segment (less than a quarter of total length) leading.

Squiggling of the trailing segment will tend to redirect the kinesin-generated forces off axis, allowing the on-axis forces between the lead and trail segments to reach a balance. The trail segment is under compression, as is evident from the tendency for loops to lift off the surface.

3.4.10 GDP-taxol mosaic microtubules which change travel directions do not necessarily glide slower

Since slowdown of microtubules by the leading minus ends may contribute to squiggling of GDP-taxol microtubules, perhaps slower-gliding microtubules in respective preparations of mosaic microtubules may also experience larger changes in travel direction.

Plotting the relationship between microtubule gliding velocity and change in travel direction revealed no clear indication that microtubules which had larger changes in travel direction moved slower, see Figure 3.19. This is especially evi-
Figure 3.18: Gliding patterns and velocities of different GDP-taxol segmented microtubules. (a): GDP-taxol segmented microtubules have different gliding trajectories. (left) pig brain microtubule leading, sinuous trajectory; and (right) β3 microtubule leading, squiggly trajectory. Magenta: pig brain microtubules; green: β3 microtubules. (b): Gliding velocities of GDP-taxol pig brain, β3 and segmented pig brain-β3 microtubules in equimolar of ADP and ATP at 2 mM each. n (mean velocity of microtubules) = 20, 18, 15 and 16, respectively, from one experiment. The inset shows gliding speed of all β3 fragment leading microtubules and the subset where short β3 segment is leading (n = 4 out of the 15 microtubules). PB MT: pig brain microtubule.
Figure 3.19: Relationship between instantaneous gliding velocity and change in microtubule travel direction. The relationship was fitted to a linear regression line for GDP-taxol microtubules with different tubulin compositions. Shaded area shows confidence interval. PB_MT: GDP-taxol pig brain microtubules, as reference. Data from Section 3.4.3 and Section 3.4.6.

Dent by GDP-taxol β3 microtubules, which were slower sliding but less squiggly than GDP-taxol mosaic microtubules. Potentially therefore, slower microtubule sliding is not the cause of squiggling. Instead squiggling of microtubules may be due to the tendency of different regions within a sliding microtubule to want to move at different speeds wherein these intrinsically faster moving region got slowed down because of various reasons, see Section 4.10.2 for discussion. Although less likely, another potential reason for causing squiggling of segmented microtubule is change of protofilament number, see Section 4.10.5.
Chapter 4

Discussion

4.1 The multi-tubulin hypothesis lives on

The idea that different tubulin isoforms do specific and different jobs in cells was suggested early in the history of microtubule biology and has evolved progressively [203–208]. Currently, much emphasis is placed on the tubulin code, usually meaning specific patterns of PTMs that are written and read [209] to adjust the properties of specific tubulin isoforms, including the recruitment of different interactors. An alternative view of the code includes the specification of the tubulin isoform as part of the code [210]. This view to some extent reconciles the multi-tubulin hypothesis with the view that PTMs are key. It includes the possibility for the core sequence of each tubulin isoform to specify different function, including different interactor-affinity, independent of any PTMs. My study interrogates the different lattice structures and dynamic instability behaviour of two different metazoan tubulin isoforms, seeking to understand differences in their conformational and dynamic responses to the allosteric effectors taxol and kinesin.
4.2 Choice of tubulin isoforms

Although it is still not clear whether specific $\alpha$ and $\beta$ tubulin isoforms tend to form dimers with each other, some $\alpha$ and $\beta$ tubulins are known to predominantly express in particular tissues. The $\beta 3$ tubulin isoform is almost exclusive to neurons. Its non-neuronal expression is linked to cancer and has been associated with taxol resistance [170] whereas the expression of $\beta 4$ tubulin is less tissue-specific. The human $\beta 2$ isoform also has rather global expression, but it has already been characterised respecting its structures and lattice stabilities as modulated by different effectors [3]. I decided to investigate zebrafish $\beta 4$ tubulin, which is only 5 residues different from its human counterpart, see Figure 4.1(a), and also highly similar to human $\beta 2$ tubulin. By comparing two different single isoform metazoan microtubules assembled from respective human $\alpha 1b/\beta 3$ and zebrafish $\alpha 1c/\beta 4b$ tubulins, where they have almost identical $\alpha$ tubulin sequences (only differ in 2 residues), I have been able to focus on the impact of sequence divergence in $\beta$ tubulins. In addition, this allows me to establish zebrafish as a tubulin model and also to get a general sense of whether a few single residue substitutions make a substantial difference to microtubule properties, by comparing my tubulins not only with each other but also in the context of previous published work on human tubulin isoforms.

Remarkably, the divergent residues at the surface of $\beta$ tubulins, especially those that differ between the $\beta 3$ and $\beta 4$ isoforms used in my experiments, tend to cluster around the interfaces formed between neighbours in the lattice, especially lateral neighbours. These interfaces include the H1-S2 and H2-S3 loops, both of which participate in interprotofilament interactions. The other main player in lateral interactions, the M-loop, is, by contrast, highly conserved, see Figure 4.1(b). For the $\alpha$ component of heterodimers, the human $\alpha 1b$ tubulin and the closest equivalent of $\alpha$ tubulin in zebrafish, $\alpha 1c$ (encoded by gene tuba1c) were chosen. These two $\alpha$ tubulins are nearly identical, having different residues at only two positions, see Figure 4.1(c).

In the near future, human $\beta 4b$ tubulin will also be investigated, to find
out whether the distinct microtubule properties I observed are due to β tubulins of different organisms used.

4.3 Effectiveness of my tagging scheme

My strategy of tagging both C-termini allows purification of only full-length tubulins, thereby avoiding truncated tubulin products. If I had used cleavable tags at the C-terminal, the remnant remaining following protease cleavage (part of its recognition sequence) would be left behind. For example, the commonly used Tobacco Etch Virus (TEV) protease cleaves between 7th and 8th residues of the recognition sequence. Following protease cleavage, the 7-residue remnant sequence would remain. This remnant is almost as big as my affinity tags, although my tags are also preceded by a 5-residue long linker (GGSGG), making the exogenous sequence 13-residue long for both α and β tubulins.

It is nevertheless useful to check for the impact of C-terminal tags by comparing with N-terminally tagged tubulin constructs that can avoid or minimise remnant residues of cleavage recognition site. However, N-terminal tag has only been reported for α tubulin [3] whereas tagging the N-terminus of β tubulin results in insoluble protein [194]. In addition, existing reported N-terminal tagged α tubulin also employed the use of a linker with two remnant residues after tag cleavage [3], which might impact microtubule polymerisation competency [62]. It may be possible to express N-terminally SUMO-tagged tubulin, which supposedly allows purification of native tubulin after removal of tag, but it has only been previously attempted for N-terminally SUMO-GFP tagged α tubulin in HEK293T cells [211] so the efficiency in Sf9 cells is still unclear.

My double-tagging scheme allows us to define both isoforms in a heterodimer. My two purified tubulin isoforms both show authentic dynamic instability, with behaviours that are clearly different, despite their identical tags. A further merit of leaving tags in place is that they can potentially be used as
Figure 4.1: Sequence alignment of α and β tubulins used in the experiments, the human equivalent of β4 tubulin also shown. (a): human and zebrafish β4 tubulins showing 5 divergent residues; (b): the β tubulins used in my experiment, human β3 and zebrafish β4 tubulins, are relatively more divergent when compared to (a), especially in the H1-S2 and H2-S3 loops; and (c): the α1 tubulin isoforms used. Identical residues are highlighted in the same darker shade whereas divergent residues are in a lighter shade.
fluorescent labelling sites in the future.

Since the success of the first single isoform tubulin purification in insect cells, several labs have employed this approach for single isoform tubulin purification which gave reasonable but different yields, potentially due to different tagging schemes, including an internal tag in α tubulin [2, 194] and cleavable N-terminally tagged α tubulin [3], see Table 1.1.

Discrepant structures have been reported for GMPCPP microtubules built of α/β3 tubulin, with mainly 13 protofilaments found for α1b/β3 microtubules by Ti and coworkers [3] but 14 protofilaments for α1a/β3 microtubules by Vemu and colleagues [2], also mentioned in Section 1.9.1. It is possible, but not established, that these differences are due to the different tagging schemes. The 13-protofilament β3 microtubules have an internal 8x histidine tag in the α tubulin acetylation loop which is involved in lateral interaction between protofilaments. Positively charged histidines placed in this loop might affect the interaction site despite the absence of obvious perturbation of microtubule functions, since acetylation of only one residue, K40, in α tubulin is already sufficient to weaken lateral interaction [61, 103].

4.4 Lattice structure and dynamics are tubulin isoform-specific

Previous work has shown that some single isoform microtubules favour certain numbers of protofilaments which associate with distinct microtubule dynamics [2, 3, 130], as mentioned in Section 1.9.1. Brain microtubules with their protofilament number tuned by drugs showed higher growth rates correlated to higher protofilament number [180]. Increased longitudinal lattice spacing tends to correlate to a more stable lattice, as suggested by the expanded lattice of GMPCPP microtubules [31]. These are all indications of correlation between microtubule dynamics and structure.
4.4.1 Different single isoform microtubules show different dynamics

In this study, dark-field microscopy was employed to investigate the dynamics of single isoform microtubules using unlabelled tubulin, thereby avoiding artefacts due to fluorophore labelling. Fluorophore-conjugation typically uses random labelling followed by cycles of polymerisation-depolymerisation to select a tubulin population capable of reversible self-assembly. This inevitably reduces the yield of functional tubulin. For this reason also, I chose to emphasise dark-field microscopy.

The GMPCPP seeds I used for microtubule nucleation in dynamics assays were assembled from pig brain tubulin instead of respective single isoform tubulins. This allows me to have a defined microtubule seed configuration as GMPCPP pig brain microtubules are known to mainly adopt a 14-protofilament structure. Using “single isoform” seeds would still inevitably require addition of biotinylated pig brain tubulin and fluorescently labelled pig brain tubulin to allow seeds to be anchored down to the glass surface and visualised. It is possible that protofilament number might be varied by mixing different tubulin isoforms, as mentioned in Section 1.9.1.

My experimental data show that single isoform β3 and β4 microtubules have rather similar growth rates when assembled in 1 mM GTP, see Figure 3.4(b). My determination of microtubule growth rates as a function of tubulin concentrations is preliminary, because more replicates and tubulin concentrations are needed for a robust plot to determine dynamics parameters more accurately, such as \( k_{on} \), \( k_{off} \) and critical concentration. The critical concentration for growth can then be obtained from the x-intercept, see Figure 3.4(b). The y-intercept is \( k_{off} \), the dissociation rate constant; and \( k_{on} \), the association rate constant, can be obtained from the gradient with Equation (3.1). When free tubulin concentration is below critical concentration, microtubules depolymerise. When the free tubulin concentration is exactly equal to the critical concentration, microtubule dynamics is in a steady state in which the overall (population) rate of dimer association equals that of dissociation. Microtubule growth is a second-
order reaction which is directly proportional to free tubulin concentration and microtubule (end) concentration, whereas classically, the shrinkage rate is independent of the free tubulin concentration. Note, however, that this view has been challenged [43].

The depolymerisation rates of β4 microtubules and β3 dynamically-unstable single isoform microtubules are more than 2× different, at 688.3 ± 217.7 nm/s and 303.3 ± 140.6 nm/s (mean ± SD), respectively, when fitted with a single Gaussian distribution. The slower depolymerisation rate of my dynamic GDP β4 microtubules, in comparison to β3 microtubules, is reminiscent of the slower rate of α1b/β2b microtubules compared to α1b/β3 microtubules in their GMPCPP states [3]. If so, microtubules that contain β3 tubulin as the β tubulin component might be intrinsically less stable regardless of the nucleotide states for GDP and GMPCPP lattices.

In apparent contradiction however, other work has shown that α1a/β3 microtubules depolymerised slower than α1b/βI+βIVb microtubules, at rates of 30.5 μm/min and 38.9 μm/min, respectively [2, 130]. Although the human βIVb tubulin has an almost identical sequence to my β4 tubulin, and is highly similar to β2b tubulin, the α1b/βI+βIVb tubulin in that study, which was isolated from a human embryonic kidney (tsA201) cell line, was made up of equal amount of βI and βIVb tubulins [130]. The properties of pure βI tubulin still remain uncharacterised. In addition, the α1a/β3 tubulin used had an internal histidine tag, located in the acetylation loop of α tubulin, which could potentially impact interprotofilament interactions unlike the native α1b/βI+βIVb tubulin isolated from tsA201 cells. Further, the α1a/β3 tubulin was obtained from insect cell expression [130], PTM states might also vary. Caution is thus needed when comparing the lattice stability of these two types of microtubules, due to different tagging schemes and potentially distinct PTMs, in addition to uncharacterised properties of βI tubulin. However, what we can infer from these experiments is that apparently slight sequence differences between different tubulins can substantially influence microtubule dynamics.
My two tubulin isoforms are from different organisms so my results might not directly reflect differences between human tubulins. Although the zebrafish β4 tubulin used here is only 5 residues different from the human version, see Figure 4.1(a), it does remain possible that these two isoforms have different properties. In the future, I will investigate whether this small difference between these two β4 tubulin isoforms contribute to different microtubule dynamics and/or interactions with MAPs. In this work, I demonstrated that apparently closely similar tubulin isoforms can nonetheless differ markedly in their microtubule dynamics and effector interactions.

4.4.2 Isoform- and effector-dependent lattice structures are recognised by other effectors

Whether or not and how tubulin isoforms can recruit different binding partners is still an enigma. There seems to be no one-size-fits-all rule for sequence-structure-function relationships even for the most fundamental microtubule activities despite the rather similar overall 3D structures and sequences of various tubulin isoforms. Most microtubules undergo some longitudinal compaction upon GTP hydrolysis (shortened dimer rises) or upon binding of some EBs, but *S. pombe* microtubules do not show longitudinal compaction associated with either of these two factors [114], also mentioned in Section 1.5.2. This demonstrates that different tubulin isoforms can have different structural responses to the same type of effector.

My own data reveal different structural responses of seemingly similar tubulin isoforms to taxol, as reflected by the kinks developed in β4 microtubules but not β3 microtubules. These distinctions lead to different GDP-taxol microtubule gliding rates. In addition, I find that kinesin-1 recognition of β3 and β4 tubulin isoforms is sensitive to the GDP-taxol lattices but not to the GMPCPP lattices, using microtubule gliding velocities as the readout, see Section 4.9.1. In this case, I propose that the kinesin senses the tubulin isoform indirectly, by sensing whether it changes spacing upon binding different nucleotide and/or drugs.
By contrast, binding of some MAPs appears less sensitive to (subtle) structural differences, for example, GFP-MCAK and chTOG-GFP bind GMPCPP α1b/β2b and α1b/β3 microtubules to similar levels, despite the structural distinctions between the usually 14-protofilament and 13-protofilament arrangements of the respective microtubules [3].

4.4.3 Altered protofilament lateral interactions underlie different single isoform microtubule assembly and dynamics

Since different single isoform tubulins can tend to assemble with different protofilament numbers and have distinct depolymerisation rates in GMPCPP [3], it is possible that protofilament number directly affects lattice stability, due to variations in hinge angle formed between protofilaments [26].

In my own work I saw that depolymerisation of the GDP lattice of single isoform microtubules can happen with two distinct rates, see Figure 3.2. This switch between two modes of linear depolymerisation behaviour could potentially be due to a change of protofilament number along the microtubule axis. Changes in the number of protofilaments and seams have been demonstrated by structural studies using purified mosaic tubulin assembled in vitro [16, 17]. Single isoform microtubules in both GDP-taxol and GMPCPP states can adopt different number of protofilaments and experience transitions in diameter along the same microtubules [3] as brain microtubules do, which suggests the possibility of transitions of protofilament numbers for a single microtubule.

Particular tubulin isoforms have been shown to prefer specific lattice arrangements. GMPCPP α1b/β2b microtubules adopt 14-3 and 15-4 lattice arrangements, forming 66% and 28% of the population, respectively, whereas their GDP-taxol lattices tend to have fewer protofilaments distributed among 12-3, 13-3, 14-3 and 15-4 arrangements [3]. In contrast, 95% of GMPCPP α1b/β3 microtubules conform to a 13-3 arrangement and nearly 80% of GDP-taxol α1b/β3 microtubules adopt a 12-3 arrangement. Both GMPCPP and GDP-taxol α1b/β2b
microtubules generally have more stable lattices (slower depolymerisation) than their counterpart α1b/β3 microtubules, supporting a role for lattice arrangement in determining microtubule stability.

Since microtubules are assembled via both longitudinal and lateral inter-dimer interactions of tubulin dimers, divergent tubulin residues located in either of these two major interfaces might in principle directly affect lattice conformation and/or binding angles and energies, and thereby microtubule dynamics.

In lateral interfaces, the H1-S2 and H2-S3 loops of one tubulin associate with the M-loop of its immediate neighbour, see Figure 4.2. Variations in microtubule conformation at the lateral interface have indeed been detected, by comparison of single isoform α1a/β3 microtubules with brain microtubules. In the GMPCPP state, the H1-H1’ loop in β tubulin was observed to be displaced approximately 3 Å away from the H1’-S2 loop [2].

![Figure 4.2](image-url)

**Figure 4.2:** Luminal view of two tubulin dimers showing lateral interdimer interactions. The interaction sites mainly involve the H1-S2 loop (cyan) and the H2-S3 loop (red) of one tubulin and the M-loop (yellow) of another tubulin. Only the lateral interaction sites of β tubulin are coloured, for simplicity. PDB: 6WVL.

Importantly, Ti et al. observed structural differences between different
single isoform microtubules with the exact same tagging scheme, excluding any
differential impact of tags on isoform-dependent lattice arrangement and sta-
bility. Two single isoform GMPCPP microtubules, α1b/β3 and α1b/β2b, were
found to have broadly distributed structural differences. In β tubulin, a >1 Å
CαRMSD (Cα root mean square deviation) was predominantly found in region
proximal to the M-loop and in the H1-S2 loop, in addition to the H11’ region
[3] which participates in longitudinal interdimer interactions [30]. Respecting
these two isoforms, all of these regions harbour divergent residues, except for
the H11’ which is conserved. Remarkably, despite the use of the same α tubu-
lin for α1b/β3 and α1b/β2b microtubules, the overall magnitude of structural
differences in α tubulin was comparable to that of β tubulin between these two
single isoform microtubules, suggesting allosteric communications between β
and α tubulin subunits [3], as previously proposed for GTP hydrolysis [30, 118]. The
major structural changes concentrated in the M-loop and the H1-S2 loop, again
highlighting the potential role of this region in regulating protofilament numbers,
and potentially lattice stability, via the making of distinct lateral interactions [3].

Motifs directly involved in microtubule interprotofilament interactions,
such as the H1-S2 and H2-S3 loops and the M-loop are more likely to directly
modulate lattice stability. The β3 and β4 isoforms used in my own experiments
differ in a total of 39 residues, see Figure 4.1(b). These divergent residues span
throughout the entire sequence, but are predominantly clustered in the CTTs
and in or adjacent to the H1-S2 loop (9-residue difference) and the H2-S3 loop
(4-residue difference). Interestingly, the M-loop is fully conserved in most β tubu-
lin isoforms, whereas β3 tubulin has the 275th serine residue replaced by alanine.
Whilst CTTs do play a role in microtubule dynamics, as suggested by removal of
CTTs after subtilisin treatment [212], it is less likely that they directly influence
the stability of the built lattice, as CTTs are unstructured and β tubulin CTTs
are distal to both the longitudinal and lateral interdimer interaction sites. Nev-
ertheless, there is evidence that the CTTs of α tubulins can affect microtubule
dynamics, possibly due to their proximity to longitudinal interdimer interaction sites [196].
Although my α tubulin isoforms were from different species, the sequences differed only by two residues which are unlikely to significantly impact microtubule dynamics due to their locations, see Figure 4.1(c).

4.5 Mixing tubulin isoforms gives rise to new microtubule properties

Previous studies have already established that tubulin isoform composition of microtubules can regulate their dynamics and structures [3, 130]. I infer that the lattice is plastic, and that mosaic (mixed-isofrom) microtubules might acquire different properties by recruiting different tubulin isoforms.

In my experiments, microtubules prepared from various ratios of β3 to β4 tubulin mixtures exhibited dynamics intermediate between those of the respective pure single isoform microtubules. At 12 μM total tubulin concentration, microtubules assembled from 1:4 β3:β4 tubulin mix had a shrinking rate between that of pure β3 and β4 microtubules, at about 450 nm/s (median rate), see Figure 3.5. In contrast, the equimolar mixture of β3 and β4 tubulins gave rise to microtubules with almost the same depolymerisation rate as pure β3 microtubules, 724.7 ± 287.2 nm/s compared to 746.7 ± 324.6 nm/s (median ± SD). This non-linear dependence of the microtubule depolymerisation rate on isoform ratio is reminiscent of observations on the growth rate in Vemu and colleagues’ work, where microtubule growth at a 1:1 molar ratio of α1a/β3 and α1b/βI+βIVb tubulins is very similar to that of pure α1a/β3 microtubules at the same total tubulin concentration [130]. Thus, mosaic microtubule properties are intermediate between but not always proportionate to the amount of soluble tubulin used for microtubule assembly. This non-linearity might suggest cooperativity/allostery in the conformational communication between specific tubulin isoforms, and/or be due to biased incorporation of particular tubulin isoforms.

In cells, it is a more complicated picture due to microtubule regulations
by various MAPs, including possible biased recruitment of tubulin isoform. Establishing the properties of pure tubulin isoforms in vitro can help clarify the picture.

4.5.1 Lattice mismatch may be responsible for non-linear mosaic microtubule dynamics

Lattice mismatch has been proposed for α1a/β3 tubulin and pig brain tubulin, as the nucleation efficiency of α1a/β3 tubulin from GMPCPP brain seeds at 6 µM of tubulin is less than that from α1a/β3 seeds with 33% and 92%, respectively, of seeds elongated [2].

Under similar conditions, brain tubulin can extend from 90% of GMPCPP brain seeds with the same 6 µM free tubulin. Interestingly, with 5% spiking of brain tubulin in α1a/β3 tubulin, the brain seed extension efficiency when compared to α1a/β3 tubulin alone can be rescued to 91% from 33% [2]. The barrier to nucleation was proposed to be lattice mismatch and this was further supported by structural data showing conformational differences between GMPCPP brain microtubules and α1a/β3 microtubules [2]. Lattice mismatch might thus bias incorporation of tubulin isoforms into microtubules, resulting in a different balance of tubulins in the lattice than in the solution.

A relationship between lattice mismatch and biased incorporation is consistent with my own experience in trying to generate segmented microtubules consisting of two single isoform sections within the same microtubule, assembled without taxol. In most, but not all cases, the first segment of microtubules depolymerised when the second tubulin isoform was introduced into the flow cell channel (data not shown). After extension of the first tubulin isoform from the seeds, followed by the introduction of another tubulin isoform, the free tubulin pool of the first tubulin isoform would be replaced by the second one. If the second tubulin isoform cannot add and incorporate into the microtubule lattice quickly, the tip of the first isoform segment would tend to lose its “GTP cap”,

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resulting in catastrophe and microtubule depolymerisation.

4.5.2 Mixing tubulin isoforms in the lattice may change their microscopic on- and off-rates

Novel responses might arise due to interactions between two different-isofrom neighbours in the lattice, either laterally or longitudinally. One might expect that in an equimolar mix of two tubulin isoforms with distinct $k_{on}$, mosaic microtubule dynamics might be dominated by the properties of the tubulin isoform with the higher $k_{on}$ due to potentially faster incorporation rate. However, this did not seem to be the case, as demonstrated previously [130]. Pure $\alpha_{1a}/\beta_3$ microtubules have a slightly slower polymerisation rate compared to pure $\alpha_{1b}/\beta_I+\beta_{IVb}$ microtubules at 6 $\mu$M consistent with the $k_{on}$ values of 1.8 dimer $\mu$M$^{-1}s^{-1}$ [2] and 3.7 dimer $\mu$M$^{-1}s^{-1}$ [130], respectively. With almost one-half the $k_{on}$ of $\alpha_{1b}/\beta_I+\beta_{IVb}$ tubulin, the growth rate of mosaic microtubules from equimolar of $\alpha_{1a}/\beta_3$ and $\alpha_{1b}/\beta_I+\beta_{IVb}$ tubulins resembled $\alpha_{1a}/\beta_3$ microtubules alone at total tubulin concentration of 6 $\mu$M. This suggests that interactions among neighbouring isoforms might generate new values of $k_{on}$ and $k_{off}$, which would result in different microscopic growth and shrinkage rates.

Variations in total tubulin concentration might also change mosaic microtubule lattice composition and dynamics. If the respective tubulin isoforms have distinct second order $k_{on}$ values then the relative incorporation rate of each single isoform tubulin will vary upon changing the total concentration of the isoform mix. The fraction of assembly-competent tubulin may also vary from prep to prep, and might depend on isoform.
4.5.3 Minus end dynamics is potentially less sensitive to tubulin isoform

The minus end is not an area of focus in my experiments, because under my conditions it is usually too short or not dynamic enough for accurate analysis. In other study, minus end growth rates of α1b/βI+βIVb and α1b/β3 single isoform microtubules were demonstrated to be very similar with a slight difference in catastrophe frequency [130]. However, catastrophe frequency of mosaic microtubules of these two tubulin isoforms grown off GMPCPP α1b/βI+βIVb seeds were not greatly affected by tuning the α1b/β3 tubulin proportion for microtubule assembly [130]. It is difficult to determine why the minus end dynamics is less sensitive than the plus end dynamics to tubulin isoform compositions as perhaps there is only mainly a particular tubulin isoform got incorporated into the lattice, hence, dynamics not modified markedly.

More in-depth studies of single isoform microtubules and mosaic microtubules, using fluorescently labelled tubulin isoforms, will be needed to shed light on this question.

4.6 Taxol effects are tubulin isoform-specific

As mentioned in Section 1.7, several studies have demonstrated that overexpression of different tubulin isoforms in cell lines and in patients is responsible for different taxol treatment responses. For example, overexpression of β3 tubulin has been associated with taxol resistance in ovarian cancer [170].

Taxol binds the luminal surface of β tubulin in microtubule near the M-loop which is close to the interdimer lateral interaction sites. Although the mechanisms by which taxol stabilises microtubules still remain elusive, in particular how it influences interprotofilament interactions, taxol-bound GDP microtubules are known to adopt an expanded lattice when taxol is added during
microtubule assembly, consistent with a causal link between lattice expansion and microtubule stabilisation [184]. Note that microtubule stabilisation is not always accompanied by lattice expansion, as exemplified by pelorusside stabilisation [184], implying other mechanisms such as strengthened interprotofilament interactions.

I observed isoform-specific responses of tubulins to taxol regarding microtubule dynamic instability, kinesin-driven microtubule gliding and copolymer formation. My data clarify the nature of the insensitivity of $\beta_3$ tubulin (in microtubules) to taxol.

### 4.6.1 Kinks generated in $\beta_4$ microtubules are a result of local expansion

Surprisingly, $\beta_4$ microtubules acquired kinks when assembled in 500 nM taxol, whereas kinks did not obviously develop at 100 nM taxol. No prominent kinks were observed for $\beta_3$ microtubules at all the taxol concentrations tested (even at 1 $\mu$M and 5 $\mu$M).

I wondered whether kinks were due to defects in microtubules or uneven conformational changes caused by taxol binding in patches to the GDP lattice. Defects of microtubules might be directly observable by electron microscopy. However electron microscopy cannot report dynamic effects, and I sought therefore to interrogate the nature of the kinks using optical microscopy. Since GDP-taxol brain microtubules are known to adopt an expanded lattice, I considered the possibility that the kinks are bends in the lattice produced by local patches of taxol-driven lattice expansion.

Previous work has established that mixed-isoform brain microtubules with taxol added during and after assembly adopt 82.3 Å and 81.8 Å dimer rises respectively, compared to GDP microtubules with no drug added at 81.5 Å [184]. If taxol used at substoichiometric concentration binds in patches, this might cause
uneven lattice expansion generating kinks. However, these values were all obtained using microtubules assembled from mixed tubulin isoforms and decorated with MAPs as fiducial markers. Kinesin-1 is known to cause lattice expansion in GDP brain microtubules [116, 117] whereas it was found to slightly compact GMPCPP brain microtubule lattices [31], see Table 4.1. Caution is needed when interpreting structural data involving the use of microtubule effectors as fiducials, to rule out the potential impacts of effectors on microtubule properties.

Table 4.1: Selected dimer rise of porcine brain microtubules from different preparations. Below are kinesin-labelled porcine brain microtubules, except for * which is kinesin-free, prepared in different conditions.

<table>
<thead>
<tr>
<th>Microtubule States</th>
<th>Dimer Rise (Å)</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP microtubules (taxol added pre-assembly)</td>
<td>82.3</td>
<td>[184]</td>
</tr>
<tr>
<td>GDP microtubules (taxol added post-assembly)</td>
<td>81.8</td>
<td>[184]</td>
</tr>
<tr>
<td>GDP microtubules (no taxol)</td>
<td>81.5</td>
<td>[184]</td>
</tr>
<tr>
<td>GMPCPP microtubules*</td>
<td>83.95</td>
<td>[31]</td>
</tr>
<tr>
<td>GMPCPP microtubules</td>
<td>83.30</td>
<td>[31]</td>
</tr>
</tbody>
</table>

To test whether the kinks I observed were due to lattice expansion, bound taxol was washed out using free tubulin solution without taxol. Indeed, kinks generated during microtubule assembly in the presence of 500 nM taxol relaxed upon taxol washout. Therefore, I conclude that kinks are indeed caused by localised regional expansion of the lattice induced by taxol binding instead of persistent defects. It is not clear why the expansion was not propagated throughout the entire lattice to evenly stretch out the microtubule as a whole.
According to previous measurements, the occupancy of taxol molecules at 500 nM on brain microtubules is about 1 taxol molecule for every 20 tubulin dimers [138]. One might assume that at this 1:20 binding ratio there should be 1 taxol molecules bound for every about 1.5 period of 13-protofilament microtubules. With such binding pattern, microtubule expansion should be somewhat homogenous. Alternatively, the kinks I observed might indicate that this low occupancy reflects high affinity binding of a few taxol molecules in patches along the microtubule lattice, and/or at tips, with little or no binding of taxol elsewhere.

Potentially, at 500 nM substoichiometric taxol concentration, the arrival of the first few taxol molecules might initiate a patch by causing local lattice expansion, producing a conformation wave of lattice expansion that recruits more taxol. This idea is also supported by the fact that no kinks were observed at lower taxol concentration at 100 nM as taxol may be too sparsely bound to elicit local expansion.

Cooperative binding of allosteric effectors to microtubules, based on effector-driven lattice expansion, has also been demonstrated by bend-locking of microtubules when kinesin-1 solution, at particular concentrations, was introduced to microtubules by hydrodynamic flow [116]. Peet et al. proposed that kinesin predominantly binds and expands the convex side of microtubules bent by the flow so microtubules remained bent after flow has stopped. In addition, kinesin-1 binds preferentially to a subset of GDP microtubules in solution, (without taxol, stabilised by addition of glycerol in the buffer), at intermediate kinesin concentration, possibly due to cooperative binding [116, 117]. This heterogeneity was not observed for GMPCPP microtubules over the range of kinesin concentrations tested, possibly due to incapability of expansion for the already-expanded GMPCPP microtubules. Shima et al. further demonstrated cooperativity of GDP microtubule conformational changes triggered by kinesin in ATP, using x-ray fibre diffraction, showing microtubule spacing of the population varies from compact conformation to heterogenous population to expanded lattice as the kinesin/tubulin dimer ratio increased [117].
If taxol binding indeed expands β4 microtubules in localised patches, the question becomes, why does expansion remain localised? One possibility is that the patch of expansion needs to centre on a lattice defect, but this is speculation. There is however evidence that fluorescent taxol analogs bind at defects, at which the microtubule protofilament number changes [180]. Since taxol washout relaxes kinks, this idea would presumably imply persistence of defects following washout. Preferential, high-affinity binding of taxol at defects would explain why the patch of taxol-driven lattice expansion does not propagate but does not explain kink relaxation, or perhaps generation of kinks requires both defects and taxol-induced lattice expansion. Nevertheless, appearance of kinks suggests that expansion was not propagated laterally as otherwise microtubules would not acquire kinks.

With only two isoforms used, I cannot yet conclude whether kinking of microtubules when assembled in the presence of taxol is the more usual behaviour amongst metazoan tubulin isoforms, or whether the no-kink behaviour I saw in β3 microtubules is more common. In the future, other human version of β tubulin isoforms will be tested, including the highly similar β2 tubulin isoforms, see Figure 4.3.

**Figure 4.3:** Sequence alignment of human β2 and β4 tubulin isoforms.
4.6.2 Taxol can drive continuous processive growth of both \( \beta_3 \) and \( \beta_4 \) microtubules

When taxol concentration was high enough, such as at 500 nM, catastrophe of dynamic microtubules (as detected by rapid shrinkage of the lattice) can be fully inhibited, provided free tubulin was available. Under these conditions, growth persists - the taxol concentration used was sufficient to increase microtubule polymer mass. Titration enables the taxol concentration to be set at a level that does this without causing nucleation of new microtubules.

In contrast, when free tubulin was washed out with buffer containing taxol at the concentration in which microtubules were assembled, microtubules depolymerised, but slowly. I hypothesise that taxol, by appreciably slowing depolymerisation following a catastrophe, supports almost immediate rescue and a return to growth. The hypothesised rescues are imperceptible.

This idea is implied by my observation of increased rescue events in \( \beta_3 \) microtubules, in the presence of free tubulin, at 250 nM taxol, during slow depolymerisation events, see Figure 3.6(a). At 100 nM taxol, \( \beta_3 \) microtubules depolymerised back to the seeds, albeit with slower depolymerisation, without rescue. Rescues for \( \beta_4 \) microtubules are not discussed here as microtubules grew processively even at 50 nM taxol.

This processive growth in the case of taxol is especially prominent at minus ends, since these do not normally exhibit substantial dynamicity in the absence of taxol with the tubulin concentration used at 12 \( \mu \)M. At 500 nM taxol, some \( \beta_3 \) microtubules show almost the same growth rates for both the plus and minus ends, see Figure 3.8(a). Most minus end growth events were not measurable when taxol was absent due to short length. Note that despite being able to convert both ends to continuous growth, taxol did not drastically alter microtubule growth rates at either end, see Figure 3.8(b). Dynamics of \( \beta_4 \) microtubules were again not determined as they became kinky at 500 nM taxol.
4.6.3 More taxol is required to suppress catastrophe of β3 microtubules potentially due to lower binding affinity

Taxol is less effective at suppression of catastrophe for β3 microtubules than β4 microtubules. 50 nM taxol was sufficient to support processive growth of β4 microtubules, whereas 500 nM taxol was needed to achieve the same effect for β3 microtubules. One could argue that this difference is related to the intrinsically faster depolymerisation rates of β3 microtubules compared to β4 microtubules. However, when depolymerisation was triggered by washing out free tubulin, β4 microtubules had a depolymerisation rate of 152.7 ± 88.1 nm/s (median ± SD) at 50 nM taxol, whilst β3 microtubules depolymerised at 108.4 ± 76.3 nm/s in 100 nM taxol, see Section 3.3.1, also discussed in Section 4.6.4.

In this case, slowing down depolymerisation of β4 microtubules to 152.7± 88.1 nm/s was already sufficient to block catastrophe (when free tubulin was present) whereas β3 microtubules with shrinking rate at 108.4 ± 76.3 nm/s still depolymerised back to the seeds. Accordingly, the k_{on} values and/or microtubule growth rates of respective tubulin isoforms may also play a role. This is relevant to the association of fast-growing microtubules with longer GTP caps, thereby increase in delay time of catastrophe after free tubulin washout [45]. However, in my case, β3 microtubules grow even slightly faster than β4 microtubules at 12 µM tubulin, see Figure 3.4. Nevertheless, the exact mechanism of catastrophe and rescue is still not yet well-understood.

The lower sensitivity of β3 microtubules to taxol could point in two directions: firstly, taxol might bind less well to β3 microtubules; alternatively, taxol might bind well but be less effective on β3 microtubules. I favour the lower affinity theory as evident by the diminished binding of β3 tubulin in brain microtubules to taxol analog, 2-(m-Azidobenzoyl)taxol, as determined by mass spectrometry and liquid scintillation counting [153].

I demonstrated directly that β3 microtubules were almost unaffected (catastrophes were still observed) by a concentration of taxol able to stabilise
β4 microtubules in the presence of free tubulin. Increasing taxol concentration from 50 nM to 500 nM, did fully stabilise β3 microtubules. This clearly favours a simple model in which taxol can fully stabilise the β3 lattice, but binds at a lower affinity. Possibly, taxol-induced lattice expansion, which occurs for β4 but not β3 microtubules, might increase the affinity of the lattice for taxol which allows relatively low taxol concentration to stabilise β4 microtubules.

4.6.4 Taxol stabilises both the β3 and β4 GDP lattices

Since β4 microtubules resisted catastrophe at 50 nM taxol, it was necessary to wash out free tubulin to determine the stability of the lattice, in terms of depolymerisation rate, at this taxol concentration.

By washing out free tubulin with microtubule assembly buffer having the same taxol concentration at which microtubules were assembled, depolymerisation rates of microtubules at particular taxol concentrations could be determined, see Figure 3.6(b). At 100 nM taxol, β3 microtubules depolymerised at 108.4 ± 76.3 nm/s (median ± SD), about 1/6 of that in the absence of taxol (688.3 ± 217.7 nm/s). At this same 100 nM taxol concentration, β4 microtubule depolymerisation was slowed down to 50.0 ± 36.1 nm/s, which is also about 1/6 of that when taxol was not present (303.3 ± 140.6 nm/s).

As mentioned in Section 1.7.4, stabilisation is not always accompanied by lattice expansion. Gliding rates of GDP-taxol β4 microtubules were 0.426 ± 0.064 μm/s and 0.672 ± 0.069 μm/s and (median ± SD), at 500 nM and 10 μM taxol, respectively, see Figure 3.10. GDP-taxol β4 microtubules at 500 nM taxol depolymerised minimally, indicating lattice stabilisation, yet, travel slower than their counterparts at 10 μM taxol, implying minimal lattice expansion.
4.7 Segments within segmented isoform microtubules respond differently to taxol

To investigate the possibility of conformational communication between regions of different tubulin isoforms along the same microtubule, I prepared segmented isoform microtubules. With microtubules grown at 12 μM in 1 mM GTP, a substantial number of microtubules with an initial single isoform β3 segment grown off GMPCPP pig brain seeds depolymerised upon the introduction of second tubulin isoform, this potentially suggests lattice mismatch where β4 tubulin nucleates less well with the use of β3 microtubule as seed template; thus, β3 microtubule loses its “GTP cap” and catastrophises. This correlates to my experience in preparing segmented microtubules for the gliding assay where pure β3 microtubules and pure β4 microtubules did not readily undergo end-to-end joining, unlike β3 microtubules and pig brain microtubules.

Therefore, segmented microtubules were assembled in low dose taxol (100 nM) to allow extension of second segment from the first segment.

4.7.1 Segments depolymerise at rates that approximate their cognate single isoform lattices

At 100 nM taxol, the depolymerisation rates of segmented microtubules composed of adjoining regions of β3 and β4 microtubules fit well to a double Gaussian distribution with rates of 210.7 ± 142.3 nm/s and 26.1 ± 21.8 nm/s (mean ± SD), see Figure 3.9(b). Individual single isoform segments in segmented microtubules depolymerised at rates that differed by approximately 2-fold from the characteristic shrinkage rates of β4 and β3 lattices within single isoform microtubules at 108.4 ± 76.3 nm/s and 50.0 ± 36.1 nm/s, respectively. See Figure 3.6(b).

Why do the depolymerisation rates of segments in segmented-isoform microtubules differ from those of their cognate single isoform microtubules? One possibility is variations between experiments in the exact amount of taxol in so-
olution. Taxol is hydrophobic so may drop out of solution over time. Another possibility is change of protofilament number of microtubules, thereby changing microtubule response to taxol. This can well be the reason for this 2-fold discrepancy in depolymerisation rates of β4 microtubules because in segmented microtubules, β4 microtubules used β3 microtubules as “seeds” whereas the single isoform β4 microtubules were grown off GMPCPP pig brain seeds. However, this cannot explain the discrepancy for β3 microtubules, because both segmented microtubules and single isoform β3 microtubules grew off GMPCPP pig brain seeds.

What is very clear is that taxol exaggerates the difference in lattice stability between segments of segmented microtubules, in terms of depolymerisation rates, when compared to difference between pure single isoform β3 and β4 microtubules from slightly over 2× (shrinking at 688.3 ± 217.7 nm/s and 303.3 ± 140.6 nm/s, single Gaussian distribution, see Figure 3.3) in the absence of taxol to almost 8× in segmented microtubules at 100 nM taxol. Thus, 100 nM taxol exaggerates an intrinsic 2-fold difference in the depolymerisation rate of the β4 and β3 lattices, increasing it (in 100 nM taxol) to approximately 8-fold.

When comparing depolymerisation rates of respective microtubule isoforms in segmented microtubules at 100 nM taxol with that of respective single isoform microtubules without taxol, the rate of β4 microtubules was slowed down by about 10× from 303.3 ± 140.6 nm/s to 26.1 ± 11.8 nm/s, whereas β3 microtubule depolymerisation rate decreased by only about 3× from 688.3±217.7 nm/s to 210.7 ± 142.3 nm/s.

4.8 Sequences distal to the taxol binding pocket control allostERIC signalling by taxol

The weaker binding of taxol to β3 microtubules could potentially be due to replacements of two residues near (but not in) its taxol-binding site, which are
conserved in most other mammalian β tubulin isoforms, but unique to β3 tubulin, namely T218A and S275A, see Figure 4.4 for sequence alignment and the locations of these two residues. The 218th and 275th residues are located at the leucine cluster and M-loop, respectively, near the taxol binding pocket. Taxol directly abuts the M-loop. The conservation of the M-loop across different tubulin isoforms is striking. In tubulins that do not bind taxol, such as S. pombe and S. cerevisiae, the M-loop sequences are more divergent. Interestingly, previous study on β tubulins, WWSSB1 and WWSSB2, of another yew tree species (Taxus baccata, as Taxus brevifolia tubulin sequences are not available), shows that both the T218 and S275 are conserved in these yew tree β tubulins [150]. They suggested the divergent residues lying in the S9-S10 loop, and some other residues in some regions, may play a role in taxol binding, thereby promoting taxol resistance in yew tree.

Another region of β tubulin which has great potential in participation of taxol activity regulation includes residues 38 to 46, constituting part of the H1-S2 loop, as this region shows greater mobility in molecular dynamics simulations [213]. Although within this region only one amino acid residue is different between β3 and β4 isoforms, another four amino acids are different in other part of this H1-S2 loop, see Figure 4.1(b).

Potentially therefore, the reduced taxol affinity of β3 tubulin might be a result of different M-loop positioning and/or mobility, caused by sequence differences that are largely (but not entirely) clustered in the H1-S2 loop. Conversely, taxol-dependent expansion of the β4 lattice may be facilitated by its H1-S2 loop – the “M-loop receptor”, and this expansion might feed back to increase taxol affinity. But these ideas are speculative and will require EM and further mutagenesis work to test them. What is clear is that although the β3 lattice has a lower affinity for taxol, and does not appear to undergo taxol-dependent expansion, it does bind taxol and it is stabilised by taxol – it is just that a (much) higher concentration of taxol is required to do this.

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Figure 4.4: Divergent residues of β3 tubulin in the leucine cluster and the M-loop compared to yeast and other human β tubulins. (a): Sequence alignment of the leucine cluster and the M-loop regions of yeast β tubulins (boxed with dashed line; TBB_yeast: *S. cerevisiae* and TBB_SCHPO: *S. pombe*) and of human β tubulins. (b): Luminal view of taxol binding pocket where two of the residues near the site are conserved in most mammalian β tubulins except for β3 tubulin. PDB: 6WVL.
4.8.1 Longitudinal allosteric signalling by taxol is attenuated

I showed that segmented microtubules depolymerised in two distinct phases in the presence of 100 nM of taxol, with no evidence for either phase influencing the depolymerisation rate of the other. This suggests minimal longitudinal transmission of allosteric signals through the microtubule lattice, see Figure 3.9(a). This was clearest in the presence of taxol, when the linear depolymerisation rate of the two adjoining segments could be tuned to be approximately $8 \times$ different, with no detectable crosstalk.

This hypothesis of attenuated allosteric communication is also supported by the observation of kinky $\beta$4 microtubules, albeit a higher taxol concentration (500 nM) was used. Supposing it is indeed longitudinal expansion that causes kinks, then the fact that kinks are localised suggests that longitudinal allosteric signalling is attenuated.

4.8.2 Lateral allosteric signalling is attenuated but can be transmitted in some cases

Likewise kinks in the $\beta$4 lattice, induced by taxol, will only happen if the lattice expansion is not being allosterically copied across to lateral neighbours. Testing these ideas will require electron microscopy.

However, lateral propagation of conformational changes may be possible for some types of modulations. Peet and colleagues showed that when GDP pig brain microtubules were anchored to a kinesin-coated surface, in the absence of nucleotide and free tubulin and taxol, depolymerisation of the entire microtubule was inhibited [116]. Since only the lower surface of the microtubules is gripped by the kinesin, yet the whole tube is stabilised, the protofilaments on the opposite side of the microtubule likely are stabilised allosterically [116]. This lateral transmission of stabilising conformational information was incomplete however. Faint trailing ends were occasionally seen for these microtubules where the top
of the microtubule had shortened back, leaving a half pipe or taper of a few protofilaments, a few microns long, adhering to the substrate behind the slowly-depolymerising tip of the microtubule. This was attributed to splitting of the lattice, induced by expansion only of the protofilaments bound to the kinesin.

The effectiveness of this lateral communication pathway might relate to variations in the “M-loop receptor” (H1-S2 loop) sequences of different single isoform microtubules, allowing β4 microtubules to be more readily stabilised by taxol, because a taxol-induced stabilising conformational shift can somehow be locally propagated, driving further binding of taxol at low dose, relative to β3 microtubules.

4.9 Microtubule gliding velocity can depend on both tubulin isoforms and effectors

I tested whether my pure single isoform microtubules interact similarly with surface-bound kinesin. GMPCPP brain microtubules have been reported to glide about 30% faster than GDP-taxol microtubules [189, 202]. To this end, microtubule gliding assays with my defined-isoform microtubules in either GDP-taxol or GMPCPP state were performed on glass coverslips coated with full-length *D. melanogaster* kinesin-1.

4.9.1 Kinesin walking senses different GDP-taxol microtubule lattices

GDP β3 and β4 microtubules glided at drastically different rates at 0.431±0.049 μm/s and 0.672±0.069 μm/s (median ± SD), respectively, at 10 μM taxol, see Figure 3.10(a). Since both β3 and β4 tubulins had the exact same tagging strategy, this difference must derive directly from intrinsic differences between the lattices of my single isoform microtubules – either directly in the
kinesin recognition surfaces of individual heterodimers, or indirectly as different lattice conformational changes induced by taxol.

Important kinesin-interacting residues of microtubules appear fully conserved between my two tubulin isoforms. Crucial charged residues on tubulin involved in kinesin-1 interaction were identified previously by using charged-to-alanine mutagenesis in α and β tubulins. Mutants of α tubulin α-E415A, -E416A, -E418A, -E421A and of β tubulin β-E410A and -D417A of yeast can modulate microtubule affinity and kinesin walking velocity [214]. Among these mutants, the α-E415A mutant (residue located in the H11-H12 loop) has the most marked k_{cat} reduction in microtubule-activated ATPase activity, showing only one-fifth of the rate of the wild type microtubules, corresponding to the slowdown of kinesin motility [214]. These residues are fully conserved in the tubulin isoforms I used, as is the entire kinesin recognition surface as judged using cryo-EM reconstructions of the kinesin-microtubule complexes, see Figure 4.5 for the divergent residues on the surface of tubulin dimers.

Consistent with this, both GMPCPP β3 and GMPCPP β4 lattices travelled at approximately 0.7 µm/s, and GDP-taxol β4 microtubules glided at a similar rate at 0.672 ± 0.069 µm/s. Clearly, GDP-taxol β3 microtubules are the outliers, sliding with a dramatically slower rate at 0.431 ± 0.049 µm/s.

Since the same residues form the kinesin-tubulin interface in both lattices, another potential reason that may cause this gliding velocity difference is perhaps different conformations of microtubules. Indeed, GMPCPP appears to induce a conformational change in this interface that causes about a 50% increase in the sliding rate of GMPCPP β3 microtubules compared to that of GDP-taxol β3 microtubules. If this conformational change corresponds to an increase in lattice spacing, then kinesin stepping would be sensing this increase.

This idea predicts that the compact β4 microtubule lattice may also show slower gliding. The absence of prominent kinks in β4 microtubules in microtubule dynamics assays at lower taxol concentrations suggests that β4 microtubules might not show lattice expansion under these conditions. If so, their
Figure 4.5: Structure of kinesin bound tubulin showing conserved kinesin binding site on tubulin. Kinesin (yellow) bound tubulin dimer from different views. The kinesin-tubulin dimer interface of β tubulin (cyan) is conserved between β3 and β4 tubulins whereas the divergent residues (magenta) locate at other sites. PDB:4HNA.
kinesin-driven sliding might be slower. For this purpose, both β4 microtubule assembly and subsequent gliding assays were performed at 500 nM taxol. As predicted, these conditions appreciably slowed sliding of β4 microtubules to 0.426 ± 0.064 μm/s with minimal depolymerisation, see Figure 3.10(b), consistent with my “combination” hypothesis, that taxol expands the lattices of β4 but not β3 microtubules, and that the kinesin stepping mechanism senses this spacing change and alters its walking rate.

Combining this result with my earlier evidence that taxol can expand the β4 lattice (producing kinks) but not the β3 lattice, see Section 4.6.1, the difference in gliding velocities I observed for β3 and β4 microtubules in GDP-taxol states may indeed derive from their distinct lattice spacings, with GMPCPP, but not taxol, able to expand the β3 microtubule lattices, and both GMPCPP and taxol able to expand the lattice of β4 microtubules. A difference in kinesin stepping rate on GDP microtubules (stabilised by capping with GMPCPP-tubulin) versus all GMPCPP microtubules was previously reported [215]. It is remarkable that kinesin can sense such a small difference in lattice spacing.

4.9.2 CTTs may also contribute to different kinesin running velocities

Is it nonetheless possible that differences other than lattice spacing could be responsible for, or contribute to, the very different sliding velocities of GDP-taxol β3 and β4 microtubules?

The negatively-charged CTTs of microtubules can influence kinesin binding through electrostatic interactions, see Figure 4.6 for β tubulin CTT location, affecting kinesin properties such as velocity and run length. The involvement of CTTs in modulation of kinesin/MAP binding is well-demonstrated by previous studies whereby microtubules with part of tubulin C-terminal sequence (around 10 amino acids for α tubulin and about 20 amino acids for β tubulin) removed by subtilisin treatment had reduced kinesin run length and velocity along with 3-fold decrease in kinesin-bound bead binding [60]. In addition, regula-
Figure 4.6: Kinesin binds intradimer interface of tubulin dimer. The resolved few residues of CTTs were highlighted in red whereas the rest were not resolved by cryo-EM. Green: α tubulin; cyan: β tubulin; and yellow: kinesin. PDB: 4LNU, nucleotide free kinesin.

ation of microtubules by kinesin can in turn be affected by microtubule CTTs as subtilisin-treated microtubules are more resistant to disassembly by kinesin-13 due to lower kinesin binding affinity [216].

More recent work has shown that divergent residues in human β tubulin CTTs may regulate kinesin velocity and run length, as demonstrated for kinesin-1, albeit with epothilone B-stabilised microtubules [63]. Unlike my data, which shows distinct gliding velocities of GDP-taxol β3 and β4 microtubules, Sirajuddin et al. found that only microtubules having C-terminal tails of β tubulin encoded by gene TUBB7 showed a significant reduction in kinesin-1 velocity, in comparison with other tubulin isoforms [63]. Interestingly, although kinesin-1 velocity along microtubules with a β3 tail was no different than most other tubulin iso-
forms, the run length of kinesin-1 was greatly reduced and this reduction could be restored when the C-terminal lysine of β3 tubulin was removed [63]. The merit of this work is that the differences observed must be contributed from the divergent β tubulin CTTs as the tubulins used in their work were a chimera of *S. cerevisiae* tubulin core and respective human tubulin CTTs, in a Δx-CTT tubulin background to avoid potential crosstalk [63].

It is unlikely that the difference in sliding rates between my GDP-taxol β3 and β4 microtubules is solely due to the divergent CTTs, because the difference is erased by switching to the GMPCPP lattices. Further, the faster, GMPCPP-like sliding rate of GDP-taxol β4 microtubules can be reduced to a GDP-taxol β3-like rate by titrating down the taxol concentration. In both cases with no change to the CTTs.

One may argue that the discrepancy observed between my experiment and the chimeric yeast-human tubulins could be due to the drug used for microtubule stabilisation. The use of epothilone B was necessary because yeast tubulin binds taxol very poorly. I have preliminary data showing that β3 and β4 microtubules stabilised by epothilone B also had distinct gliding velocities at 0.392 ± 0.037 µm/s and 0.542 ± 0.103 µm/s (median ± SD), respectively, see Figure 3.15 top two panels. The gliding velocity difference was not as dramatic as taxol if solely based on the median gliding rate. However, the velocity of GDP-epothilone B β4 microtubules showed a very broad distribution which was potentially caused by mixed populations of microtubules, as further discussed here for taxol in Section 4.9.6. Thus, it is unlikely that MSAs used caused this discrepancy.

Nonetheless, it would be interesting to investigate whether the constructs with the same tubulin core but different CTTs (chimera of β4 tubulin core with β3 tubulin CTT versus full-length β4 tubulin) can override the huge difference in gliding velocities observed for my two single isoform GDP-taxol/GDP-epothilone B microtubules in the future. On the other hand, using MSAs, such as peloruside, that do not cause lattice expansion may also help an-
swering whether kinesin indeed recognises these two single isoform microtubules in GDP-taxol state through lattice spacings. Both experiments would test my current hypothesis that the kinesin walking mechanism discriminates between different GDP-taxol lattices by reading their lattice spacing.

4.9.3 Kinesin can recognise different drugged GDP microtubule lattices

Combined my findings and Sirajuddin’s work, it is likely that the different gliding velocities I observed for GDP-taxol β3 and β4 microtubules are due to different lattice spacings that can be recognised by kinesin. Previous reports show that kinesin-1 can discriminate between GDP-taxol and GMPCPP microtubule lattices to cause preferential binding [116, 117, 190]. There is also evidence that mixed-isoform brain GMPCPP microtubules glide faster than their GDP-taxol counterparts [189, 202].

My results indicate that different gliding velocities for different GDP-taxol microtubules most likely originate from their different microtubule lattice conformations. Firstly, perhaps most obviously, the slower β3 microtubule gliding rate only holds true in GDP-taxol state with gliding rate at \(0.431 \pm 0.049\) µm/s whereas in GMPCPP state, the β3 microtubules glided at \(0.705 \pm 0.044\) µm/s (median ± SD), see Figure 3.10(a) and 3.13(a). Although the GDP-taxol β3 microtubule dimer rise has yet to be reported, its dimer rise in GMPCPP state was demonstrated to be comparable to GMPCPP pig brain microtubules [130]. In addition, both GMPCPP α1b/β3 and α1b/β2b microtubules adopt similar expanded lattices with dimer rises of 82.11 Å and 81.75 Å [3], respectively. All of these findings correspond to the similar gliding rates of β3, β4 and pig brain microtubules in GMPCPP state in my experiment, further discussed in Section 4.9.7. This subtle structural difference between the GMPCPP lattices of different isoforms is apparently not recognised by kinesin-1, whereas differences in the GDP-taxol lattices are recognised.
More directly, I found that the GDP-taxol β4 microtubules glided at different rates depending on the taxol dose at $0.426 \pm 0.064 \mu m/s$ and $0.672 \pm 0.069 \mu m/s$ at 500 nM and 10 µM taxol, respectively. The dose of taxol could possibly modulate microtubule lattice structure. This idea was supported by my microtubule dynamics assay in taxol where kinks were only observed at 500 nM taxol but not 100 nM. Combined all these observations, it is likely that kinesin-1 directly identifies different GDP-taxol single isoform microtubule lattices resulting in different microtubule gliding speeds.

The distinct gliding rates of different GDP microtubule isoforms stabilised by epothilone B could also potentially be caused by distinct lattices induced by the drug, see Figure 3.15. Although there are no structural data reported for mammalian microtubules stabilised by epothilone B, yeast S. cerevisiae microtubules stabilised by this drug adopt a more expanded lattice compared to the GTPγS counterpart (generally believed to be in GDP.P₁ state which adopts a more compact lattice than GMPCPP microtubules), albeit the dynamic microtubules also have an expanded lattice [120], as discussed in Section 1.5.2. Although there is no direct evidence showing that epothilone B may block microtubule compaction upon GTP hydrolysis, thereby preserving the expanded lattice arrangement, combined my GMPCPP and GDP-taxol data, the lattice of GDP-epothilone B β4 microtubules might also be more expanded, leading to faster gliding on the kinesin-coated surface.

Notably, stepping of full-length single Drosophila kinesin molecules under load on epothilone-stabilised single isoform S. pombe GMPCPP microtubules was appreciably slower and showed a higher stall force compared to epothilone-stabilised mixed-isoform brain GMPCPP microtubules [215]. This suggests that yeast and mammalian tubulins can drive kinesin to step at different speeds even when nominally in the same conformational state (with potentially similar lattice spacing).

Note also that kinesin-1 can itself cause microtubule lattice expansion [116, 117]. It is unclear whether lattice expansion caused by taxoids and lattice
expansion caused by kinesin reflect similar or different conformational changes. If the underlying conformational changes are similar, then my data suggest kinesin does not expand the lattice of β3 microtubules and β4 microtubules in low dose taxol regime in gliding assay, since if it did, they would go faster.

4.9.4 Brain microtubules with taxol added pre- or post-assembly glide at different rates, consistent with differential lattice expansion

Previous studies show more than 30% increase in gliding velocity for brain GMPCPP microtubules than GDP-taxol microtubules [189, 202]. I repeated this and did not observe such a big increase in velocity for GMPCPP pig brain microtubules, but rather only about a 10% increase. In addition, only a subtle difference in gliding velocity was observed between GMPCPP and GDP-taxol microtubules for β4 isoform when taxol was used at 10µM whereas more than 50% increase was found when compared to β4 microtubules prepared at 500 nM taxol, potentially due to different lattice structures as discussed in Section 4.9.3.

Since microtubule conformation could potentially be modulated by different taxol concentrations, as implied by kinks generated in β4 microtubules and by different microtubule gliding rates, I asked whether taxol added at different stages of microtubule preparation could result in different gliding velocities, see Figure 3.14. Previous work found different lattice spacings for brain microtubules with taxol added during or after microtubule assembly, see Table 4.1 in Section 4.6.1 [184].

To check this point, pig brain microtubules were prepared using 40µM tubulin in GTP, to generate “seed” templates, then further diluted 20× or 2× in taxol solution to have a final concentration of 10µM taxol. The reaction with 2× dilution was deemed to have taxol present during microtubule assembly as there was still plenty of free tubulin available to be driven into microtubule by
taxol. In contrast, the reaction with 20× dilution was treated as microtubules with taxol added post-microtubule assembly, as only a very low concentration of free tubulin remained available for further microtubule elongation/assembly.

Indeed, when taxol was present during microtubule assembly, about 95% of microtubules glided at 0.646 ± 0.065 µm/s with a much smaller population at 0.479 ± 0.023 µm/s (mean ± SD, fitted with a double Gaussian distribution), see Figure 3.14(a). The fast and slow rates roughly correspond to the gliding rates of GDP β4 microtubules at 10 µM taxol/GMPCPP β4 microtubules and of GDP β4 microtubules at 500 nM taxol/GDP β3 microtubules at 10 µM taxol, respectively.

For brain microtubules with taxol added post-microtubule assembly, two gliding rates were again observed, with 67% of population peaked at 0.456 ± 0.060 µm/s and a smaller population at 0.634 ± 0.063 µm/s (mean ± SD), see Figure 3.14(b). Because there might still be a small population of microtubules formed upon addition of taxol, I do not rule out that the population ratio might shift by adjusting the dilution factors of the tubulin mix with taxol solution, for example, higher dilution factor makes less free tubulin available to assemble into microtubule by taxol; potentially producing a larger population of slow gliding microtubules.

The difference in gliding velocities for brain microtubules with taxol added during and after microtubule assembly is in agreement with structural data where microtubules have shorter dimer rise when taxol is added post microtubule assembly (81.8 Å) compared to that of microtubules assembled in taxol (82.3 Å) [184]. This dependence on when taxol is supplied also corresponds to my dynamics experiment (preliminary data, not shown). Assuming taxol can expand the microtubule lattice spacing when added after microtubule assembly, the β4 microtubules with kinks should be able to “stretch out” when microtubules are supplemented with more taxol. However, under dark-field microscope, β4 microtubules with kinks (microtubules assembled in 500 nM taxol) did not straighten up when supplemented with 10 µM taxol. This is again consistent with structural
data and further support the idea that microtubule/tubulin respond differently depending on whether taxol is made available during or after assembly.

Note that my data at 10 𝜇M taxol might slightly overestimate and underestimate the gliding velocities of respective β3 and β4 GDP-taxol microtubules, due to the presence of 5% fluorescent porcine brain tubulin. GDP-taxol pig brain microtubules in my assays had gliding velocity of about 0.65 𝜇m/s. The incorporation efficiencies of the respective tubulins are still unclear, however, substantially different incorporation of fluorescently labelled mixed-isoform brain tubulin into β3 and β4 GDP-taxol lattices is implausible, because the fluorescence was similar.

4.9.5  Might my tagging scheme affect β3 and β4 microtubule gliding velocities?

The histidine and FLAG tags of my recombinant tubulin could potentially interfere with kinesin running behaviours to certain extent. The impact of this exact same tagging strategy has been studied in greater detail previously with the tagged single isoform yeast microtubules, mutated to bind taxol, found to glide 5% faster than their counterparts without tags [194]. Therefore, the impact of tags on microtubule gliding velocity could be real when compared to tagless microtubules, but minimal. Nevertheless, my β3 and β4 microtubule gliding assays should still be directly comparable due to my two defined-isoform heterodimers using the exact same tagging strategy. The actual impact of the tags on microtubules can only be tested after tag cleavage in the future.

Other than lattice spacing differences, the cause of the difference in sliding velocities of GDP-taxol single isoform microtubules could be divergent residues in sites distal from the kinesin-microtubule interface. Besides sequence variations in CTTs and in the H1-S2 loop involved in interdimer lateral contacts, there are a few divergent residues in the tubulin core, which could possibly affect kinesin interactions, despite the main kinesin interaction site at the tubu-
lin intradimer interface appearing to be identical for my two tubulin isoforms. Allosteric effects involving these different residues in the tubulin core could be partially responsible for the very substantial differences I observed in sliding velocities.

Another potential reason for the discrepancy in kinesin-driven microtubule sliding velocities for \( \beta_3 \) and \( \beta_4 \) microtubules between my experiment and Sirajuddin’s study is the kinesin constructs. In their work, C-terminally GFP tagged truncated kinesin was used to run along microtubules attached to glass surface through biotin-streptavidin linkages, whereas full-length kinesin was used in my gliding experiment by non-specific adsorption to the glass surface. Both constructs however are dimeric, so that microtubule sliding is expected to report the walking behaviour of individual dimeric molecules. Both full-length and truncated dimer constructs should track the protofilament axis. A further difference is that I used full-length \textit{Drosophila} kinesin, whereas they used human kinesin \cite{63}.

Combining all these pieces of evidence and my GMPCPP and GDP-taxol microtubule gliding experiments, it is more likely that slower gliding rate of GDP-taxol \( \beta_3 \) microtubules was due to the lack of a structural response to taxol at the kinesin-tubulin interface of \( \beta_3 \) tubulin.

4.9.6 The broad distribution of gliding velocity for GDP-taxol mosaic microtubules could be due to selective tubulin isoform incorporation

Since GDP-taxol \( \beta_3 \) and \( \beta_4 \) microtubules showed distinct gliding rates, I wondered whether the gliding velocity would shift with microtubules made of a mosaic of \( \beta_3 \) and \( \beta_4 \) tubulins.

The gliding velocities of mosaic microtubules increased with an increasing fraction of \( \beta_4 \) tubulin used for microtubule assembly, see Figure 3.11 top panel. However, for microtubules made up of 1:4 \( \beta_4: \beta_3 \) tubulin mixture (20%
$\beta 4$ tubulin), the median gliding velocity ($0.424 \mu m/s$) was almost the same as that of microtubules assembled from pure $\beta 3$ tubulin ($0.431 \mu m/s$, the presence of 5% fluorescent tubulin was neglected), albeit the difference was statistically significant. This might suggest cooperative/preferential incorporation of particular tubulin isoforms into microtubule lattices. For example, microtubule assembly from 1:4 $\beta 4: \beta 3$ tubulin mixture could potentially have only 10% of $\beta 4$ tubulin present in the lattice which may only cause negligible effects. This potential for biased recruitment was also discussed in Section 4.5, relating to its possible influence on dynamic instability.

In addition, microtubules assembled from 1:1 $\beta 4: \beta 3$ tubulin mixture had a median gliding rate of about $0.476 \mu m/s$ but show a wide distribution with potentially two populations of microtubules, fitted to a double Gaussian distribution, gliding at $0.441 \pm 0.042 \mu m/s$ and $0.567 \pm 0.054 \mu m/s$ (mean $\pm$ SD), see Figure 3.11 bottom left. There are two main reasons which may cause this wide distribution, one is individual microtubules glide at constant but different rates, another is some microtubules vary their gliding speeds from time to time. Figure 3.12 shows that individual mosaic microtubules indeed glided at relatively constant rates.

As a result, perhaps these mosaic microtubules form heterogeneous populations in which some microtubules, or some parts of some microtubules, contain tubulin isoform ratios different from that of the free tubulin mixture used for microtubule assembly. This may support the idea of cooperativity that like attracts like, with each tubulin isoform tending to interact more easily with an identical neighbour. Resolving this will require that we establish fluorescent tagging of single isoform tubulins.
4.9.7 Kinesin does not much discriminate GMPCPP \(\beta_3\) lattices from GMPCPP \(\beta_4\) lattices

I showed that kinesin may recognise GDP-taxol \(\beta_3\) and \(\beta_4\) microtubules as distinct lattices due to their differential structural response to taxol.

Could kinesin-1 discriminate between the potentially similar structures of GMPCPP microtubules of \(\beta_3\) and \(\beta_4\) isoforms? GMPCPP \(\alpha_1b/\beta_3\) microtubules and \(\alpha_1b/\beta_2b\) microtubules (highly similar to my \(\beta_4\) tubulin, assuming my \(\beta_4\) microtubule also adopts a similar structure) have similar dimer rises at 82.11 Å and 81.75 Å, respectively [3]. Thus, I employed a different nucleotide, GMPCPP, to study how kinesin recognises the conformational changes of single isoform microtubules induced by different effectors.

If both of my GMPCPP microtubules have a similar dimer rise and kinesin-1 does not discriminate against expanded GMPCPP microtubules regardless of tubulin isoforms, the gliding velocity should be about the same. Indeed, as predicted, in GMPCPP states, both \(\beta_3\) and \(\beta_4\) single isoform microtubules glided at more or less the same rate at 0.705\(\pm\)0.044 \(\mu\)m/s and 0.694\(\pm\)0.069 \(\mu\)m/s (median \(\pm\)SD), respectively, see Figure 3.13(a), although the difference is statistically significant (p < 0.0001, Mann-Whitney U test). This corresponds to more than 50% increase in gliding velocity for GMPCPP \(\beta_3\) microtubules compared to GDP-taxol counterpart. On the contrary, GMPCPP and GDP-taxol \(\beta_4\) microtubules did not experience drastic change in gliding speeds. This lack of difference in gliding velocities could well be explained by expanded lattices in both microtubules. However, I do not rule out lattice expansions induced by GMPCPP and taxol are caused by different mechanisms yet kinesin responds to the expanded lattices in the same way (faster gliding velocity) regardless of the exact conformational changes.

I also tested the sliding of GMPCPP microtubules in the presence of taxol. Overall, GMPCPP microtubules glided at more or less the same velocities with or without taxol. However, I noticed that \(\beta_4\) microtubules in the presence
of taxol occasionally became stuck onto kinesin surface. Of these stuck microtubules, some got trapped for the entire imaging duration (normally around 5 minutes) whereas some microtubules managed to escape and carry on gliding smoothly, see Figure 3.13(b). Interestingly, this has not been reported in previous literature using bovine brain GMPCPP microtubules with or without taxol for kinesin-1 running, albeit single molecule assay was performed instead [217]. This tendency to become stuck appears isoform specific. On the same surface at the same time, β3 microtubules did not become stuck.

Although it is not clear why GMPCPP β4 microtubules got stuck in the presence of taxol, it could be associated with the counteracting actions between slight compaction of GMPCPP microtubules by kinesin [31, 189] and reverse/blocking of microtubule compaction by taxol.

In summary, kinesin does not respond to the GMPCPP lattices of β3 and β4 microtubules by showing dramatic rate difference in gliding.

4.9.8 Kinesin walking rate is determined by tubulin sequence, lattice conformation and lattice geometry

The rate of ATP hydrolysis by unloaded wild type kinesins-1 is directly coupled to the mechanical stepping rate. Therefore, faster kinesin walking should correspond to increase in ATPase activity. This was inferred from the 36% increase in kinesin velocity, accompanied by 32% faster ATP hydrolysis rate, observed for GMPCPP brain microtubules compared to GDP-taxol brain microtubules [189]. This correlation between ATP hydrolysis rate and kinesin velocity is especially evident in gliding assays using the yeast microtubules built of α tubulin mutant E415A where the ATPase activity was significantly reduced, so was the gliding velocity, compared to wild type microtubules due to the important role of α-E415 as the only critical catalytic residue for kinesin ATP hydrolysis [214].
However, this direct coupling can be broken. For example, kinesin under load can consume ATP molecules but fail to make a step which results in futile ATP hydrolysis. ATP hydrolysis cycle of kinesin is a multi-step process and in principle any step in the cycle might limit how fast kinesin can walk along microtubules. Therefore, hopefully by studying microtubule-activated ATPase activities of kinesin by β3 and β4 microtubules can address the mechanism of how different GDP-taxol microtubules differ in gliding velocities in the future.

Just as MAPs can regulate microtubule dynamics, kinesin-microtubule interactions can also be regulated by MAPs. Kinesin processivity can be inhibited by both 3RS and 4RL-tau isoforms, of which the inhibition was only observed for GDP-taxol microtubules but not GMPCPP microtubules although tau binds microtubules of both types to similar extent [217]. This again suggests that kinesin-microtubule interaction can potentially be modulated by microtubule effectors.

It is as yet unclear how tau differentially modulates the conformation of different lattices to affect kinesin velocity and run length, but these observations nonetheless strengthen the case that kinesin reads the microtubule lattice and responds by varying its walking velocity and run length.

### 4.10 Sinuosity of microtubule sliding driven by kinesin is dependent on tubulin isoform composition and nucleotide state

Why do GDP-taxol brain microtubules sometimes run in circles or travel in snake-like trajectories, whereas GMPCPP microtubules travel as straight rods across the surface, see Figure 3.16(a) and 3.17? Unexpectedly, GDP-taxol single isoform β3 and β4 microtubules travelled in rather “straight” trajectory, see Figure 3.16(b), unlike pig brain microtubules as reported and observed in my experiments.
I exploited the intrinsic gliding behaviours of GDP-taxol brain microtubules and GDP-taxol single isoforms microtubules to try to answer this question. GDP-taxol segmented microtubules and mosaic microtubules were prepared to study how microtubules would behave in the situations where microtubules composed segments with intrinsically distinct gliding velocities, compared to microtubules built from mixture of tubulin isoforms.

4.10.1 In sliding segmented microtubules, a slow-sliding leading segment causes squiggling of a faster trailing segment

Since microtubules of different isoforms travelled at different speeds, I asked how microtubules composed of different isoform segments would behave. For this purpose, GDP-taxol pig brain and β3 microtubules were mixed and incubated overnight in the presence of taxol to allow spontaneous end-to-end joining.

These segmented microtubules behaved unexpectedly. One might think that when the leading end is fast-moving (pig brain tubulin or β4 tubulin segment) with slow end trailing (β3 tubulin segment), microtubules might break at the junction. Whereas if the slow-moving end is leading, the fast end might catch up and cause buckling at the junction. Instead, segmented microtubules exhibited distinctively different gliding patterns depending on the leading ends. When the slower gliding β3 microtubule was leading, the trailing pig brain microtubules segments often squiggled, see Figure 3.18(a). This squiggling event can be explained by the self-generated compressive force experienced by the intrinsically fast-gliding lagging microtubules which were slowed down by the slow-moving leading segments. This is evident from the fact that GDP-taxol segmented microtubules glided at rates intermediate between separate β3 and pig brain microtubules, regardless of the isoform of the leading segment, see Figure 3.18(b). Squiggling was exaggerated in mixtures of ATP and ADP. ADP-kinesin has lower binding affinity for microtubules and is incapable of walking, but can tether microtubules while ATP-kinesin molecules perform power strokes.
to move microtubules forward.

The gliding trajectories of segmented microtubules with the faster gliding pig brain microtubules leading and the β3 segments trailing remained relatively usual with occasional snake-like trajectory observed, just like GDP-taxol pig brain microtubules do.

Segmented microtubules prepared from β3 and β4 microtubules exhibited similar squiggling behaviour when the fast-moving β4 segment was led by slow-gliding β3 segment. In the opposite case, segmented microtubules of this kind normally followed relatively “straight” trajectory as most GDP-taxol single isoform microtubules did. However, the populations of segmented β3-β4 microtubules observed were smaller than pig brain-β3 microtubules. This reduced tendency for segmented β3 and β4 microtubules to form could be a consequence of incompatible ends. Such lattice mismatch has been discussed relating to the elongation of β3 microtubules from GMPCPP pig brain seeds [2], also mentioned in Section 4.5.1. If so, the tendency for segmented pig brain-β3 microtubules to form might be due to the higher heterogeneity of pig brain microtubule ends, allowing joining of β3 microtubules which also composed 5% fluorescently labelled porcine tubulin for visualisation under fluorescent microscope.

4.10.2 Sinuosity of gliding GDP-taxol microtubules requires a mixture of tubulin isoforms

I quantified the sinuosity/snake-like behaviour of gliding microtubules by comparing the angles of gliding trajectory formed by the leading tip of each microtubule at time $t_n$ and $t_{n+1}$ to that at $t_{n+1}$ and $t_{n+2}$. If a microtubule barely changes its gliding direction, this angular difference would be close to zero.

I find that snaking and spooling can be reconstituted by mixing two tubulin isoforms in GDP-taxol state. This causes a larger angular change of gliding trajectory compared to pure isoform microtubules, especially with the
equimolar mixture of $\beta_3$ and $\beta_4$ tubulins, see Figure 3.16(c), where there was a larger population of microtubules with even greater angular change compared to GDP-taxol pig brain microtubules.

Interestingly, although the gliding speed of the 20% $\beta_4$ mosaic microtubules were slower than that of GDP-taxol pig brain microtubules, the angular change of these two types of microtubules were somewhat similar despite the different gliding velocities at $0.424 \pm 0.055 \mu m/s$ and $0.645 \pm 0.069 \mu m/s$ (median ± SD) for the GDP-taxol 20% $\beta_4$ mosaic and pig brain microtubules, respectively. This suggests that the snake-like trajectory may not have direct correlation with slower gliding of microtubules as a whole.

Perhaps the most obvious reason for these elusive gliding behaviours has already been suggested by segmented microtubule gliding patterns wherein squiggling episodes can be triggered when there is significant slowdown of one part of the microtubule whilst other regions try to go faster but to no avail due to the presence of slow-gliding leading end as a “roadblock”. It is possible that the occasional change of gliding direction of single isoform microtubules (with 5% fluorescent porcine brain tubulin) and the more drastic difference in mosaic microtubules is due to the presence of respective “pure” isoform islands which gives rise to these squiggles. However, if this proves to be true, $\beta_3$ microtubules would be travelling in an even “straighter” trajectory (smaller angular change) than measured here as the fluorescently labelled pig brain tubulin used may introduce artefacts since GDP-taxol $\beta_3$ microtubules travelled significantly slower than GDP-taxol pig brain microtubules.

Although slowdown by the leading end of a microtubule might trigger squiggles, there is no strong correlation between slower gliding and change in microtubule travel direction, see Figure 3.19, which reconciles with the observation for GDP-taxol 20% $\beta_4$ mosaic microtubules and GDP-taxol pig brain microtubules as mentioned previously. Perhaps the snake-like trajectory is not caused by a microtubule which moves at a uniformly-slow speed along the longitudinal axis. Instead, squiggling/spooling may be caused by different parts of
a microtubule trying to go at different speeds but fail to do so. Note that the analysis for angular change is limited by some microtubules which formed very tight loops (overlapping loop within a single microtubule) where the advancing tip cannot be tracked.

In addition, these microtubules overall do not have strong preference in gliding clockwise or anticlockwise at any one moment, see Figure 3.16(d). However, whether a single microtubule has a preference to glide in a particular direction most of the time is not discussed here.

4.10.3 Most GMPCPP microtubules have straighter trajectories compared to GDP-taxol microtubules

Gliding GMPCPP pig brain microtubules generally followed straighter trajectories than their GDP-taxol counterparts. However, the β4 microtubules appear to be the exception as the GDP-taxol microtubules changed their direction less than the GMPCPP microtubules. Nevertheless, both GMPCPP and GDP-taxol β4 microtubules still followed relatively straight trajectories compared to GDP-taxol pig brain microtubules/mosaic microtubules, see Figure 3.17.

4.10.4 Flexural rigidity may play a role in microtubule gliding patterns

The straight trajectory of GMPCPP pig brain microtubules observed previously compared to GDP-taxol microtubules could be partly contributed by the flexural rigidity of the respective lattices.

The persistence length of GDP-taxol microtubules is about 0.6 mm which is one-third of GMPCPP microtubules at 1.8 mm whereas the double-stabilised GMPCPP microtubules with taxol did not significantly differ from just GMPCPP alone [218]. Others have also demonstrated that GMPCPP mi-
Microtubules are indeed more rigid than GDP-taxol microtubules with persistence length even up to 14.5 mm for GMPCPP microtubules [219–221], albeit with different methods used. Therefore, lower flexural rigidity could be partly responsible for the snake-like trajectory of GDP-taxol mosaic microtubules. However, most of these measurements were performed using mosaic microtubules with no kinesin bound so it is unclear whether kinesin can significantly differentially impact microtubule lattices in different nucleotide states.

There are data suggesting taxol-stabilised GDP microtubules are more rigid than GDP microtubules [219], although most experimental data and molecular dynamics simulation [222] suggested otherwise. The methods of measuring persistence length may be relevant. Length-dependent persistence length of microtubules have been observed in some experimental data [221] but not the others which could potentially originate from the methods used to obtain the measurements.

Combining my observations and previous studies, snake-like sliding of GDP-taxol pig brain and mosaic microtubules are dependent on both microtubule nucleotide states and tendency of different regions in individual microtubules to go at different speeds (different intrinsic gliding velocities but eventually get slowed down by slower-gliding regions to reach a force balance). Firstly, GDP-taxol pig brain/mosaic microtubules may contain patches of β3 tubulin which slow down gliding of some regions of the same microtubule. Secondly, pure GMPCPP β3 microtubules glide at a similar rate to GMPCPP β4 microtubules, in other words, the gliding speed difference between my two single isoform microtubules is abolished when GMPCPP is used for microtubule assembly.

4.10.5 Might a change of protofilament number promote squiggling?

A possible alternative explanation for microtubule snaking and spooling could be the number of protofilaments as 13-protofilament microtubules usually have their protofilaments parallel to the microtubule axis whereas microtubules
of 14-protofilament have skewed protofilaments with respect to the microtubule axis. In addition, cross-sections of GDP-taxol brain microtubules are more ellipsoidal than drug-free GDP brain microtubules [184]. Perhaps microtubule lattice configurations also contribute to these complex microtubule gliding behaviours.

It seems possible that squiggling can happen when a microtubule adopts different protofilament numbers along the lattice together with slowdown of microtubule gliding. If a microtubule has regions composed of 13 and 14 protofilaments, supposed for respective GDP-taxol β3 and β4 segments, torque might generate in the β4 segment due to lattice skew. While the microtubule glides along kinesin-coated surface, torque might not be able to be released in the β4 segment which eventually slowly folds into loops as the leading end is “fixed (not rotatable)” by the junction connecting the 13-protofilament region. In addition, slowdown of gliding velocity by the slow-moving β3 segments might exaggerate this situation.

However, this seems to be a less likely explanation as intuitively these squiggles or loops would have to change their frequency or pattern while microtubules are advancing, perhaps by closing the end-to-end distance, as torque accumulates. In fact, segmented microtubule gliding pattern appeared to be somewhat consistent without drastic change although not quantified. In addition, this does not seem to explain the absence of squiggle when β4 segments are leading or maybe again slowdown of gliding is required. Nevertheless, cryo-EM data would be needed to confirm this.

Recent cryo-EM data also indicate a tendency of GDP-taxol microtubule wall to switch between high curvature and low curvature states, corresponding to changes in the hinge angle between pairs of neighbouring protofilaments, to cause “distortion” [223]. In addition to drug induced microtubule “distortion”, microtubule binding domain of dynein, DNAH7, has also been reported to flatten the angle between protofilaments [224].

As a result, we could not rule out that lattice heterogeneity may cause different parts of gliding microtubules to want to go at different speeds. Early
negative stain EM data for curved gliding microtubules suggest the possibility of kinesin-induced conformational changes of lattice subunits, in mixed-isoform, taxol-stabilised microtubules [225].

4.10.6 Transient minus end tip bundles may drive squiggling

Since squiggling events appear to require one major determining factor which is a patch or patches wanting to move slower within the same microtubules which eventually make other parts travel slower than their intrinsic fast rates, as exemplified by GDP-taxol segmented microtubules, perhaps defective microtubule tips may cause sinuous microtubule sliding.

In fact, microtubules under cryo-EM show a range of different tip structures from blunt end to open sheet to a frayed or ram’s horn structure. Light microscope images provide direct evidence for longitudinally splitting at the tips of sliding microtubules, where the split fragments (protofilament bundles) are capable of gliding, albeit with slower speed and high curvature compared to closed microtubules [226]. Thus, “defective” minus end tip structures in mosaic isoform, taxol-stabilised GDP microtubules might be the dominant cause of sinuous gliding patterns.
Chapter 5

Conclusion

Microtubule structures, dynamics, mechanics and responses to effectors can be modulated by tubulin isoforms, despite the relatively small differences in the primary sequences of β3 and β4 tubulins. Due to these differences, mixed-isoform lattices gain emergent properties.

I show that GDP β3 microtubule is less sensitive to taxol in two respects. Firstly, higher taxol concentration is required to suppress β3 microtubule catastrophe. Secondly, GDP β3 and β4 microtubules potentially exhibit different structural responses to taxol as demonstrated by kinks in β4 microtubules but not β3 microtubules, by dramatically slower gliding of GDP-taxol β3 microtubules on kinesin-coated surfaces, by fast gliding of GMPCPP microtubules of both isoforms and by taxol-dependent gliding speeds of β4 microtubules. I hypothesise that taxol-dependent expansion of the GDP-taxol lattice occurs in β4 but not β3 microtubules. Microtubule sliding by Drosophila kinesin senses this difference between β3 and β4 isoforms. Further, I showed that sinuosity (squiggling) of gliding GDP-taxol microtubules can be caused by slowdown of microtubules due to difference in intrinsic gliding velocities of different regions within the same microtubules, as directly demonstrated by my GDP-taxol segmented microtubule experiment.
In any particular lattice, both lateral and longitudinal allosteric signalling appear heavily attenuated, allowing local lattice expansion (in patches), thereby causing microtubule distortion (kinks). In other words, expansion is confined in a particular region and not conveyed across the lattice, or kinks would not happen. I saw no evidence for allosteric communication between axially-neighbouring segments of segmented isoform microtubules as otherwise squiggles would not have been seen in the segmented microtubules.

Despite my demonstration that different single isoform microtubules interact differently with effectors such as taxol and kinesin, the hanging questions are the molecular mechanisms underlying the lower sensitivity of β3 tubulin to taxol and how kinesin can recognise the expanded and compact microtubule lattices. Hopefully by performing mutagenesis for tubulin isoforms in conjunction with structural studies by cryo-EM, we can further our understanding of the roles of more single isoform tubulins.
Chapter 6

Future Plans

6.1 Cryo-EM for structural studies

The structures of my single isoform microtubules remain unanswered. Until now, the difference in lattice spacings of GDP-taxol $\beta_3$ and $\beta_4$ microtubules is still a speculation. Hopefully, cryo-EM can provide necessary structural information about these microtubules.

6.2 Tag-free tubulin and human version of $\beta_4$ tubulin

Up till this point, the tubulin isoforms generated are double-tagged with 8x histidine and FLAG tags at the C-termini of $\alpha$ and $\beta$ tubulins, respectively. CTTs are exposed on the surface of microtubules, therefore, the presence of tags could potentially affect interactions of microtubules with MAPs although these tagged $\beta_3$ and $\beta_4$ microtubules are still capable of showing distinct microtubule dynamics and gliding behaviours on kinesin-coated surface. In the future, cleavable sites will be introduced to investigate and compare the impact of tags and
to make the tubulin isoforms more representative of the native tubulin.

In addition, to make the case more relevant to human, human equivalent of β4 tubulin will also be generated. Both human and zebrafish isoforms are expected to give similar results due to only 5-residue difference, if not, this will also give new insights into how with just a few residues could cause distinct microtubule behaviours. Another interesting isoform to look at is β1 tubulin as it is the most divergent one among the mammalian β tubulins and it is highly expressed in platelets which is associated with the formation of marginal bands of platelets.

6.3 Fluorescent labelling of tubulins and generation of tubulin mutants

The exact composition of tubulin isoforms getting incorporated into the lattice is unknown. Fluorescent labelling of single isoform tubulin with different colours will allow visualisation and potentially reveal dynamics of respective tubulin isoform incorporation. To do so, the current tagging scheme can be exploited, for example, by using Ni-NTA-Atto conjugates to label the 8x histidine tag. Fluorescent taxol will be revisited but used alongside the non-fluorescent one, say in 1:9 ratio, as microtubules were not stabilised well by 100% fluorescent taxol in our hands. Pull-down assay using anti-tubulin antibody could also potentially provide information about respective isoform incorporation efficiency. However, the use of antibodies has to be handled with care as some antibodies have been reported to bind unintended tubulin isoforms. Alternatively, unnatural amino acid approach may be used to allow site-specific labelling of tubulin [227].

In terms of studies of molecular mechanisms of taxol, tubulin mutants can be generated to decipher the underlying causes of differential responses of β3 and β4 microtubules to taxol. To begin with, the 218th alanine in β3 tubulin can
be replaced by threonine which is otherwise conserved in other human $\beta$ tubulin isoforms to investigate the effects of this unique residue. The presence of alanine in place of threonine is believed to associate with lower taxol affinity for $\beta_3$ tubulin [153, 228]. In addition, swapping the H1-S2 loop sequences, which is a relatively divergent region between these two tubulin isoforms, might reveal why these two single isoform microtubules have distinct dynamics and response to taxol.

It is also interesting to study the effects of taxol on the already expanded microtubule lattice such as mutant that is incapable of undergoing GTP hydrolysis, the $\alpha$-E254A mutant. For example, $\beta_4$ microtubules with this mutation might fail to further expand their lattice to develop kinks or cannot be further stabilised by taxol. If taxol can further stabilise and expand this mutant microtubule lattice, it might suggest the involvement of interprotofilament interactions.

6.4 **Synergistic effects of microtubule effectors**

Kinesin-1 has been demonstrated to cause lattice expansion and stabilisation [116, 117]. Fluorescent taxol has higher affinity for GTP-/GMPCPP-tubulin in microtubule lattice potentially due to the expanded lattice [182]. Perhaps taxol and kinesin can facilitate binding of each other. For example, kinesin may preferentially bind the kinks of $\beta_4$ microtubules induced by taxol to cause synergistic stabilising effects on microtubules.

In addition, taxol potentially has higher affinity for microtubule ends since this region contains “GTP cap” which is widely believed to adopt a more expanded structure than microtubule shaft. This might also become relevant to the case whereby fluorescence taxol accumulates at the defects of microtubules as these sites are more accessible to taxol in solution [180].
6.5 Microtubule-kinesin interactions

Since different single isoform GDP-taxol microtubules show different gliding velocities across kinesin-coated surface, the binding affinity and force generation of kinesin can be studied at single-molecule level to gain more understanding of kinesin-single isoform microtubule interactions.

In addition, microtubule-activated ATPase activity of kinesin can be investigated to decipher the mechanism of differential kinesin velocity along different microtubule isoforms.

Last but not least, some kinesins/kinesin mutants do not appear to recognise microtubule spacing. Exploiting these motors in motility assays in the future can potentially provide extra piece of evidence that kinesin-1 used in my experiment does recognise different GDP-taxol microtubule lattices.
References


84. Iftode, F., Clérot, J.-C., Levilliers, N. & Bré, M.-H. Tubulin Polyglycyla-


131. Ti, S.-C., Pamula, M. C., Howes, S. C., Duellberg, C., Cade, N. I., Kleiner, R. E., Forth, S., Surrey, T., Nogales, E. & Kapoor, T. M. Mutations in Human Tubulin Proximal to the Kinesin-Binding Site Alter Dynamic In-


Supplementary information

S.1 Supplementary Movie 1

Kinks developed in β4 microtubule lattices over time with tubulin assembled in the presence of 500 nM taxol. The kinks can be relaxed upon taxol washout with free tubulin without taxol. Available from: https://doi.org/10.6084/m9.figshare.19614546.

S.2 Supplementary Movie 2

Segmented microtubules assembled from regions of pure pig brain tubulin and pure β3 tubulin exhibit a various gliding patterns. Those with slow-gliding β3 segment leading joined by the fast-gliding pig brain segment always have squiggles in the trailing part. Magenta: pig brain microtubule segment; green: β3 microtubule segment. Available from: https://doi.org/10.6084/m9.figshare.19665225.
S.3 Supplementary Movie 3


S.4 Supplementary Movie 4


S.5 Supplementary Movie 5

Higher population of GDP-taxol mosaic microtubules assembled from equimolar β3 and β4 tubulins glide in snake-like trajectory or in loop. Available from: https://doi.org/10.6084/m9.figshare.19665261.
Appendix

A.1 Amino acid sequences of $\alpha$ and $\beta$ tubulins including linkers and tags

The amino acid sequences of tubulin isoforms used are as follow with linkers and tags shown:

Human $\beta3$

MREIVHIQAGQCGNQIGAKFWEVISDEHGIDPSGNYVGDSDLQLERISVYYNEASSHKYV
PRAILVDLEPGTMDSVRSGAFGHLPFRPNDIFQGQSGAGNNWAKHYEGAEVLDSVLDVV
RKECENCDELQGFLTHSLGGGTGSGMTLLISKVREEYPDIMNTFSVVPSPKVDSTVV
EPYNATLSIHQLVENTDYTECIDNEALYDICRTLKATPTGLDNLNLSATMSGVTTSLS
RFPQQLNADLRKLAIVMPFPRLHFFMPGFAPLDRTAGSQQRALTVPQMDKANMM
AACDPRHGHRLTVATVFRMSSMKEVDEQMLAISKNSSYFVEWIPNVKVAVCIDIPPRG
LKMSSTFIGNSTAIQELFKRISEQFTAMFRKAFHWTGEMDEMFTEAENMNDLVS
EYQQYQDATAEEEEMYEDDEESEAQGPKGSQGDYKD

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Zebrafish β4b

MREIVHLQAGQCNQIGAKFWEVISDEHIDPTGSYHGDSDLQLDRINVVYNEATGGKYV
PRAVLVDLEPTGMDVSRGPSFGQIQPRDNVFGQSGAGNNWAKGYTEGAELVDSDVDV
RKEAESCCLQGFQLHLGGGTGSGMGTLLISKIREEYPDRIMNTFSVPSPKVSVDVV
EPYNTAVLSVHQLVENTDETIDCIDNYDICRFTPQKLTTPGVDLNLHLSATMGTVTCL
RFPGQNLADLRKLANPVPFRPHFFMPGFAPLTSRGFSQQTVRALTQMQFDANMM
AADCPRHGYLTVAAVFRGRMSMKVDQMLNVQKNSSVFVEWIPNVKTAVCDIPPRG
LKMATAFGSTAIQELFHKRISEIQFTAMFRKAFHVLWTDFEGMDHFTEAESNMNLDVS
EYQQYQDATAEEEGEEEGEEELAGGSGGKYDDDK

Human α1b

MRECISIHVGQAGVQIGNACWELYCLEHGIQPDQGMPDSKTIGGDSSFTFFSETGAGK
HVPRAVFDLEPTVIDEVTRTGYRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDLVD
RIRKLADQCTGLQFLVHSFGGTGGFTSLLMEPSYVGFKEKFDLSQYAMLSPAPQVSTA
VVEPNISLHTHTTLEHSDFCAFMVDNEAIDIYCRNLDDLERTPTYTNLNLISQIVSSITA
SLRFDGALNDLTFQNLVPYPIRHFPLATYPVASKAHEQLSVAEITNACFEPA
QMVKCDPRHKYMCCCLLYRGDVVPDKVNAIAITKTKRIQFQVDWCPFTGFKVGINYQPP
TVVPGLDLAKVQRAVCMSNTTAEAEWARLDHDFDMKAFVHLYWGEMLGEEFSE
AREDMAALEDYEEVGVDSVEGESEEEEGEEYGSSGHGHHGHGH

Zebrafish α1c

MRECISIHVGQAGVQIGNACWELYCLEHGIQPDQGMPDSKTIGGDSSFTFFSETGAGK
HVPRAVFDLEPTVIDEVTRTGYRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDLVD
RIRKLADQCTGLQFLVHSFGGTGGFTSLLMEPSYVGFKEKFDLSQYAMLSPAPQVSTA
VVEPNISLHTHTTLEHSDFCAFMVDNEAIDIYCRNLDDLERTPTYTNLNLISQIVSSITA
SLRFDGALNDLTFQNLVPYPIRHFPLATYPVASKAHEQLSVAEITNACFEPA

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### A.2 List of Primers

Table S.1: Primers used for verification of tubulin gene insertion into bacmid:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 forward</td>
<td>GTTTTCCCAGTCACGAC</td>
</tr>
<tr>
<td>M13 reverse</td>
<td>CAGGAAACAGCTATGAC</td>
</tr>
</tbody>
</table>