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The roles of testis-specific [beta]2-tubulin in drosophila melanogaster spermtail axoneme morphogenesis

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**THE ROLE OF TESTIS-SPECIFIC β 2-TUBULIN IN *DROSOPHILA*
MELANOGASTER SPERMTAIL AXONEME MORPHOGENESIS**

Thesis

Submitted to

The College of Arts and Science of the
UNIVERSITY OF DAYTON

In Partial Fulfillment of the Requirements for

The Degree

Master of Science in Biology

By

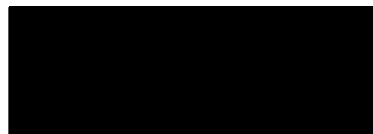
Nicole M. Humston

UNIVERSITY OF DAYTON

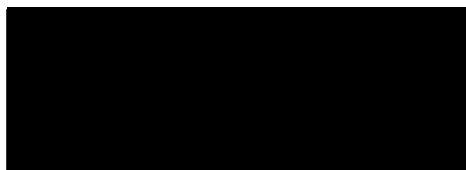
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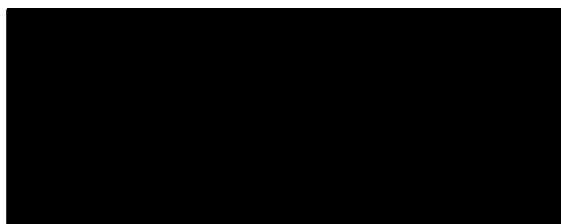
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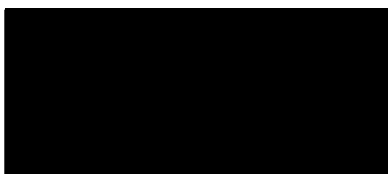
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ABSTRACT

How do amino acids work together to construct a functional protein? This question is addressed by using the role of the *Drosophila melanogaster* testes-specific $\beta 2$ tubulin protein in generating the spermtail axoneme as a model. Previous research has shown that the workhorse tubulin, $\beta 1$ cannot replace $\beta 2$ function. The two proteins differ only by 25 amino acids, therefore the ability to make a functional axoneme must lie within those 25 different amino acids. The goal of this study was to identify the $\beta 2$ specific amino acids required to make a functional axoneme.

In this study we test the hypothesis that Cys29 is indeed the key amino acid required to complete the synergism with Thr55 and Ala57. To test this hypothesis, site-directed mutagenesis was performed on a previously tested chimeric $\beta 1$ - $\beta 2$ tubulin gene, TGAR (Nielsen et al. 2001) to change the codon for amino acid 29 from $\beta 1$ Gly to $\beta 2$ Cys. The chimeric $\beta 1$ - $\beta 2$ gene was cloned into a P-element vector, then injected into flies and incorporated into their germline, such that the progeny of injected flies expressed the chimeric $\beta 1$ - $\beta 2$ tubulin protein. Genetic crosses were performed to generate a fly in which the chimeric protein was its sole source of beta tubulin in axoneme morphogenesis. These axonemes were almost full-length (2mm) compared to the 10um axonemes supported by the TGAR chimeric tubulin (Nielsen et al. 2001), which reveals that Cys29 does participate in a synergism with amino acids Thr55 and Ala57. However, the axonemes were not full-length, and the flies were not fertile, thus additional, $\beta 2$ -specific residues are necessary for axoneme assembly.

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CHAPTER I.

REVIEW OF LITERATURE

Individually, amino acids have simple chemical properties, but together they form proteins, large macromolecules with complex biological functions. How do amino acids work together to form functional proteins? This can be revealed through an experimental approach, by testing amino acid function, and by an evolutionary approach, as amino acids that work together likely evolve together. These questions are being addressed using the *Drosophila melanogaster* spermtail axoneme as a model. Axonemes are (relatively) simple structures, so a detailed analysis of the structure/function relationship between spermtail proteins and the axoneme is possible. Moreover, the assembly of a functional axoneme is sensitive to the identity of its protein components. Thus, axoneme assembly provides an excellent system for the evaluation of amino acid interaction and constraint.

AXONEMES

The axoneme is a cytoskeletal organelle that mediates the motility of cilia and flagella in all eukaryotic organisms. It is fundamentally composed of tubulin (70%), which generates an evolutionarily conserved 9+2 axoneme ultrastructure, consisting of nine doublet microtubules surrounding a central pair of singlet microtubules (Figure 1). In addition to tubulin, over 250 polypeptides (Dutcher, 1995) comprise accessory structures that work to bend and release the microtubule scaffolding, resulting in motility.

The most obvious and important of these accessory structures are the radial spokes and the dynein arms. The dynein arms bind and release adjacent doublet microtubules in an ATP-dependent manner, resulting in flagellar beating (Gibbons, 1981). The doublets slide distally to each other using the energy provided by the hydrolysis of ATP by the dynein arms. This sliding leads to the bending of the flagella, which is modulated in part by interactions between radial spokes and central pair projections (Warner and Satir, 1974).

Most research on the molecular basis of axoneme function uses the unicellular green algae *Chlamydomonas reinhardtii* as a model. *Chlamydomonas* is an ideal organism to study the axoneme because a genetic approach is possible, and its two flagella can easily be detached and recovered in highly purified fractions (Luck, 1984). Luck and his collaborators discovered a number of the proteins that comprise substructures of the axoneme, and determined that their assembly is interdependent. Single protein mutations have been identified that result in deletions of the major accessory structures including the radial spoke (Piperno et al., 1977; Huang et al., 1981) and the inner and outer dynein arms (Huang et al., 1979). By comparing results of 2-dimensional gel electrophoresis (O'Farrell, 1975) from both wildtype and mutants, Luck et al. were able to determine the different polypeptides that make up these two structures.

One example is the function of the *pf*14 mutation on radial spoke assembly. In the *pf* 14 mutant a single amino acid mutation resulted in the loss of 17 polypeptides that comprise the radial spokes (Huang et al., 1981). In the *pf* 1 and *pf* 17 mutants 5 of the 17 polypeptides were missing (polypeptides r 1, 4, 6, 9, and 10);

these comprise the radial spokehead. Luck et al. confirmed that the single amino acid mutation caused a mutation in only one polypeptide. This indicates that the polypeptides used in the assembly of the radial spokes and spokehead work interdependently of each other, therefore a mutation or loss of one polypeptide alters its interaction with the others, and the radial spokes or spokehead cannot be made.

Luck et al. also found mutations in *Chlamydomonas* that resulted in the loss of either the inner or outer dynein arm. The axonemal proteins from these mutants were compared to wildtype axonemes using 1-D and 2-D gel electrophoresis. After looking at *pf* 13A (outer arm deficient) and *pf* 23 (inner arm deficient) mutations, it was clear that each structure was composed of different polypeptides (Huang et al., 1979). By comparing the polypeptides from wild-type axonemes of four different dynein ATPases to those of the *pf* 13A and *pf* 23 it was concluded that the outer arm is a complex of two dynein ATPases 12S and 18S (Piperno and Luck, 1979, 1981; and Pfister et al., 1982). It is still uncertain how 10-11S dynein and 12.5S dynein are utilized within the inner arm (Luck et al., 1984).

The assembly of the components of the axoneme requires that proteins synthesized in the cell body travel to the growing tip of the axoneme, which is distant from their source. This occurs through a process called intraflagellar transport (IFT) and involves motor proteins and protein-carrying vesicles called rafts. IFT was originally identified in *Chlamydomonas* by Kozminski and colleagues (1993), and is the bi-directional movement of large proteinaceous rafts along the flagellum in the space between the axoneme and flagellar membrane (Figure 2).

Studies done in *Chlamydomonas*, *Tetrahymena*, *Caenorhabditis elegans*, echinoderms, and the mouse show that kinesin-II is the motor protein that mediates IFT (Kozminski et al., 1993, 1995; Morris and Scholey, 1997; Nonaka et al., 1998; Brown et al., 1999; Marszalek et al., 1999; Takeda et al., 1999). Kinesin-II is a heterotrimeric kinesin that contains two unique motor subunits from the KIF3A/3B or KRP85/95 kinesin subfamily (Cole et al., 1993, 1998; Yamazaki et al., 1996; Signor et al., 1999b). These two subunits associate with a nonmotor subunit called kinesin-associated polypeptide (KAP3) and help regulate the binding of the rafts (Yamazaki et al., 1996).

While kinesin-II regulates the anterograde transport, another motor protein, cytoplasmic dynein 1b regulates the retrograde transport of axoneme proteins back to the flagellar base, for growth or maintenance (Figure 2) (Pazor et al., 1999; Porter et al., 1999). Studies of *C. elegans* (Signor et al. 1999a) show that kinesin-II can also be cargo. It is shuttled back to the basal body by cytoplasmic dynein 1b during retrograde IFT. By this logic, it stands to reason that cytoplasmic dynein 1b is carried by kinesin-II to the tip of the flagella, but this still remains to be proven (Cole, 1999).

While kinesin-II mediates IFT in a number of organisms, it is not used in assembly of the motile *Drosophila* spermtail axoneme. Studies by Han et al. (2003) show that while mutations in kinesin-II disrupt the assembly of the non-motile sensory cilia found in chortodonal organs, they have no effect on the motile, spermtail axoneme. This indicates that *Drosophila* spermtail axoneme morphogenesis does not require IFT. There are two possible reasons for this. The first is that during spermatogenesis spermatids are not enclosed by individual plasma membranes.

Rather there is one cytoplasm for all cells, and therefore the growing tip of the axoneme is accessible from the cytoplasm (Tates 1971; Fuller 1993; Han et al., 2003). Second, *Drosophila* spermtails are extremely long, from 2.0mm in *D. melanogaster* to over 5 cm in *D. bifurca* (Pitnick et al., 1995). The components of IFT may not be able to travel this far and so another, as yet unknown, mechanism may have evolved to support this structure.

MICROTUBULES

The main building blocks of the axoneme are microtubules. Microtubules are ubiquitous cytoskeletal elements found in all eukaryotes. There are two types: axonemal and cytoplasmic. Axonemal microtubules make up the axonemes of flagella and cilia, are used in kinesin and dynein-mediated vesicular transport and are important in cell motility. Cytoplasmic microtubules are fundamental components of mitotic and meiotic spindles, are involved in organizing cell shape and help govern the location of various organelles (Becker et al., 2000).

Alpha and beta tubulin are the primary structural components of microtubules. These bind together to form a stable tubulin heterodimer. In *Drosophila* microtubules, these heterodimers bind head to tail to form a protofilament, and 13 protofilaments associate laterally to form the microtubule (Figure 3). Microtubules are highly dynamic, polar structures with a growing (+) end crowned by beta tubulin and a shrinking (-) end initiated by alpha tubulin. They have the ability to switch rapidly between growing and shrinking phases (Figure 4). This phenomenon is

known as dynamic instability and is essential to microtubule function (Mitchison and Kirschner 1984; Horio and Hotani 1986).

To begin microtubule assembly, γ tubulin is required (Oakley et al., 1990). It is located in the microtubule-organizing center, which is in the centrosome. Gamma tubulin forms the γ tubulin ring complex (γ TuRC) to which $\alpha\beta$ - heterodimers bind. The γ TuRC is also responsible for orienting the microtubule so that the growing end is distal to the microtubule-organizing center (MTOC) (Zheng et al., 1995). GTP is also required. Both α and β bind a molecule of GTP. The GTP on α is non-exchangeable, (not hydrolyzed) but is exchangeable on β . After the $\alpha\beta$ heterodimer is added to the growing end of the microtubule, the GTP on β is hydrolyzed to GDP. Hydrolysis occurs when the next subunit is added and α binds to the exposed β . This changes the conformation of the + end of the microtubule, and decreases the strength of the lateral contacts between protofilaments. When polymerization is occurring rapidly, the subunits are being added to the tip faster than the GTP they carry can be hydrolyzed. This maintains a GTP cap on the end of the microtubule. The GTP cap stabilizes the growing microtubule by strengthening lateral interactions, thus preventing depolymerization (McNally, 1999). Occasionally, the GTP molecule at the end will hydrolyze before the next subunit is added. GDP is now exposed and the microtubule is destabilized. Depolymerization occurs and the microtubule begins to shrink rapidly. This is also known as catastrophe. The GDP-bound tubulin subunits join the free tubulin pool in the cytosol, and exchange the GDP for GTP. They are then ready to be assembled into another growing microtubule.

There are proteins in the cell known to regulate microtubule polymerization dynamics. They work by stabilizing or destabilizing the + end of the microtubule. Examples of destabilizers include Op18 and XKCM1. Op18 seems to be able to work in two ways. The first way involves the sequestering of the free tubulin in the cytosol. By binding the free tubulin in the cytosol, Op18 slows the growth rate of the microtubule because there isn't enough tubulin available for rapid polymerization (Curmi et al., 1997; Jourdain et al., 1997). This will cause depolymerization. The second way Op18 works is by increasing catastrophe frequency, by stimulating GTP hydrolysis at the plus end cap. This results in beta tubulin-GDP complex at the end of the microtubule, resulting in loss of the strong lateral contacts within the protofilament and catastrophe (Larsson et al., 1999). A second destabilizer is XKCM1, a kinesin-related protein isolated from *Xenopus* eggs (Walczak et al., 1996). Recent work shows that XKCM1 does not promote catastrophe using directed motility as first thought (Desai et al., 1999). Rather than using ATP hydrolysis to translocate along the microtubule, it binds to the microtubule end and causes a conformational change that mimics GTP hydrolysis and destabilizes it (Mandelkow et al., 1991).

Other proteins are known which stabilize microtubules. MAPS (microtubule-associated proteins) bind to the outside of the microtubule in a nucleotide-insensitive manner (Desai and Mitchison, 1997). Much research has been done on the classical MAPs such as MAP1, MAP2, and *tau* in neurons and MAP4 in non-neuronal cells (Hyams and Lloyd, 1994). The neuronal MAPs weakly increase polymerization rates, strongly suppress catastrophes and promote rescues (Dreshel et al., 1992, Pryer

et al., 1992, Trinczek et al., 1995). While classical MAPS stabilize microtubules by binding to them, another MAP, XMAP215, works a rather unique way.

XMAP215 was first identified in *Xenopus* eggs (Gard and Kirschner, 1987); a human homolog is also known (Charrasse et al., 1996). It is a member of a highly conserved family of proteins called DisI and affects microtubule dynamics differently than the classical MAPs (Vasquez et al., 1994). It strongly increases the polymerization rate of tubulin at the plus end, and has the ability to increase the rate of rapid depolymerization and decrease rescue frequency, thereby increasing microtubule turnover (Desai and Mitchinson, 1997). This ability of XMAP215 to differentially affect both ends of the microtubule suggests a unique mode of action that may play an important role *in vivo* where microtubules need the ability to both grow and shrink rapidly, i.e., in the spindle during chromosome segregation (Desai and Mitchinson, 1997).

There is evidence that MAPs are the targets of signalling pathways that regulate microtubule dynamics via phosphorylation and dephosphorylation, by cell-regulated kinase/phosphatase systems (Drechsel et al., 1992; Trinczek et al., 1995; Biernat et al., 1993). The phosphorylation of MAPs by MARKs (microtubule affinity-regulating kinases) detaches the MAP from the microtubule, thus increasing instability and catastrophe (Drewes et al., 1998). The mechanism of MARK action is unknown, though it has been suggested that they are phosphorylated by another, upstream kinase (Drewes et al., 1998).

TUBULINS

This research focuses on the role of $\beta 2$ tubulin in the microtubule component of the *Drosophila melanogaster* axoneme. Together the major, $\alpha 84B$ tubulin isoform and the testis-specific $\beta 2$ -tubulin isoform comprise 70% of the mass of the spermtail axoneme. Their 3-dimensional structures have also been solved (Nogales et al. 1998, 1999), which provides valuable insight into how the protein functions. The crystalline structure of α and β are nearly superimposable, with only small variations in the lengths of some loops, slight displacement of secondary structures, and side chain densities (Figure 5) (Nogales et al., 1998). The structure consists of 2 β -sheets of 6 and 4 strands flanked by 12 α -helices, and can be divided into three functional domains: The N-terminal domain which binds the GTP nucleotide, the intermediate or drug-binding domain, and the C-terminal domain, which is the binding site for MAPs and motor proteins (Nogales et al., 1998).

The N-terminal domain consists of amino acids 1-205 and contains a Rossman fold (Nogales et al., 1998). This fold is typical of nucleotide binding proteins and consists of parallel β strands alternating with α helices. In tubulin there are 6 parallel β strands (S1-S6) alternating with helices H1-H6 (Lowe et al., 2001). T1-T6 are the loops that join each β strand to the α helix and they, along with helix H7 are important in nucleotide binding (Lowe et al., 2001). One loop in particular, the T5 loop, varies greatly between the α and β subunits. This loop is important in the binding of the nucleotide ribose and in forming longitudinal contacts along protofilaments, and seems to be responsible for the reversibility of the monomer-monomer and dimer-dimer contacts seen in dynamic instability (Lowe et al., 2001).

The difference in sequence between α and β may also explain the difference in nucleotide hydrolysis, i.e. the E- site on β is hydrolyzed (exchangeable) while the N- site on α is non-exchangeable.

The drug-binding domain consists of residues 206-381 and contains a mixed β sheet (S7-S10) and 3 surrounding helices (H8-10) (Nogales et al., 1998). The S7-H9 loop, also known as the M loop, interacts with loop H1-S1 and helix 3 of the N-terminal domain and is important in lateral interactions between protofilaments (Lowe et al., 2001). It is important to note that it is the lateral contacts that are disrupted during depolymerization of the microtubule. The M loop and regions involved in lateral contacts are known to be the least conserved between species and between α and β . These differences in sequences explain the differences in dynamic instability and the variability that occurs in protofilament number (Lowe et al., 2001).

The C-terminus consists of residues 382-446 and helices H11 and H12. These two helices are anti-parallel to each other and overlay the other domains to form the outside surface of the microtubule (Nogales et al., 1998). They are known to be the binding site of MAPs and various motor proteins and the site of many post-translational modifications (Nogales et al., 1998). The loop connecting H11 to H12 is involved in the interaction with the next monomer along the protofilament (Nogales et al., 1998). The C-terminal portion of the protein is not well characterized because it is highly variable, acidic and disordered in the zinc-stabilized anti-parallel protofilament sheets used to determine its 3-dimensional structure.

One relevant fact about the function of the C-terminus in axoneme-generating β -tubulins is that it is necessary for making the central pair microtubules (Nielsen et

al., 2001). This was discovered in a series of experiments, initiated to test the multi-tubulin hypothesis - are the multiple, *Drosophila* tubulin isoforms functionally distinct, or interchangeable (Raff 1994)? In the first experiment, the $\beta 1$ isoform, which is the major isoform used in most cell types in both males and females, was expressed in place of the $\beta 2$, testis-specific isoform. $\beta 1$ generated a short, non-motile, 9+0 axoneme (Figure 6) (Raff et al., 2000). As the $\beta 1$ and $\beta 2$ proteins are 95% identical in amino acid sequence (Figure 7), the ability of the $\beta 2$ protein to make an axoneme must reside in the 5% of residues that differ. The function of 14 of the 25 residues that comprise the 5% difference were tested in four $\beta 1$ - $\beta 2$ tubulin chimeric gene constructs, the most $\beta 2$ -like of which (TGAR, Nielsen et al. 2001) was 99% similar in identity to $\beta 2$ tubulin. Two carboxy terminus residues, Glu431 and Gly432, restored the central pair, and ten of the remaining 12 resulted in longer, $\beta 2$ -like axonemes, but were unable to restore motility to the axoneme (Nielsen et al., 2001).

Surprisingly, the remaining two exchanges, Thr55 and Ala57, actually made $\beta 1$ less able to support an axoneme. This indicates that there are other, $\beta 2$ -specific amino acids required for the proper function of $\beta 2$ Thr55 and Ala57. One amino acid, Cys29, may be key to completing the $\beta 2$ -specific interaction with Thr55 and Ala57. Cys29 is in direct contact with Thr55 and Ala57 in the folded protein, and along with Ala57, it has amino acid identity found in no other beta tubulin than $\beta 2$ (Figure 8) (Nielsen et al. submitted).

In this study we test the hypothesis that Cys29 is indeed the key amino acid required to complete the synergism with Thr55 and Ala57. To test this hypothesis,

site-directed mutagenesis was performed on a previously tested chimeric $\beta 1$ - $\beta 2$ -tubulin gene, TGAR (Nielsen et al. 2001) to change the codon for amino acid 29 from $\beta 1$ Gly to $\beta 2$ Cys. The chimeric $\beta 1$ - $\beta 2$ gene was cloned into a P-element vector, then injected into *Drosophila* embryos and incorporated into their germline, such that the progeny of injected flies expressed the chimeric $\beta 1$ - $\beta 2$ tubulin protein. Genetic crosses were performed to generate a fly in which the chimeric protein was its sole source of beta tubulin in axoneme morphogenesis. These axonemes were almost full-length (2mm) compared to the 10um axonemes supported by the TGAR chimeric tubulin (Nielsen et al. 2001), which reveals that Cys29 does participate in a synergism with amino acids Thr55 and Ala57. However, the axonemes were not full-length, and the flies were not fertile, thus additional, $\beta 2$ -specific residues are necessary for axoneme assembly.

Chapter II.

The Role of Testis-Specific β 2-Tubulin Amino Acid 29 in *Drosophila melanogaster* Spermtail Axoneme Morphogenesis.

Introduction

What is the driving force behind evolution? Charles Darwin's syllogism of evolution by natural selection states that:

1. genetic variation underlies phenotypic variation
2. resources are limiting such that a competition occurs among phenotypes, and organisms with the best phenotypes reproduce, passing their genes onto the next generation.

This results in evolution.

Which plays the greater role in phenotypic evolution? Is it the rate of mutation that gives rise to novel phenotypes (1), or the resultant competition between phenotypes (2)? Most research in evolutionary biology focuses on the second premise. For example, the paradigm of population genetics assumes genetic (allelic) variation exists first, and that this variation determines its behavior under various influences (migration, mutation, natural selection, etc. – the causes of evolution). This paradigm holds for many genes, for example, in *Colias eriphyle*, there are 6 alleles of PGI (phosphoglucose isomerase), all of which convert glucose-6-phosphate into fructose-6-phosphate. Selection on allelic variants provides a basis for glycolysis to evolve.

However, not every gene exists in multiple alleles. For example, there is little interspecific, let alone intraspecific, variation among histone homologs, due to the importance of their function (Graur and Li 2001). $\beta 2$ tubulin, the *Drosophila* testis-specific isoform used in axoneme construction provides a second example more amenable to study, as mutants only affect testis phenotypes and so are recoverable. Recent work has shown that $\beta 2$ has not evolved at a single amino acid site in over 60 million years (Nielsen et al. submitted). Why hasn't $\beta 2$ evolved? Is it due to the action of premise 2): *Dm* $\beta 2$ is an ideal phenotype and therefore maintained by selection against potential allelic variants? Or is it due to the action of premise 1): *Dm* $\beta 2$ is the only protein configuration able to make a *Drosophila* spermtail?

There is experimental evidence suggesting that $\beta 2$ is the only way to make a *Drosophila* axoneme. In tests of $\beta 2$ function, the major $\beta 1$ isoform was expressed in place of $\beta 2$. Despite being 95% identical to $\beta 2$ in amino acid sequence, $\beta 1$ could not replace $\beta 2$ function (Figures 6 and 7). Additional tests of $\beta 2$ amino acid function were performed by exchanging $\beta 2$ identity into the $\beta 1$ protein, at 14 of the 25 residues that varied between them (Nielsen et al. 2001). The most $\beta 2$ -like chimeric $\beta 1$ - $\beta 2$ protein tested was 99% similar to $\beta 2$, made functional spindles and cell-shaping microtubules, but could not generate a motile axoneme. This reveals that a very specialized protein is required for axoneme morphogenesis in *Drosophila*.

Functional tests were done to answer the question: can an alternate form of $\beta 2$ be made with the ability to generate a motile spermtail? First tested was $\beta 1$ tubulin, the workhorse protein which supports microtubules and spindles in the embryo throughout development (Kaltschmidt et al., 1991). $\beta 1$'s ability to support a

functioning axoneme was tested by expressing it in place of $\beta 2$ (Raff et al., 2000). The $\beta 1$ and $\beta 2$ tubulin proteins are 95% identical, differing in only 25 amino acids, yet $\beta 1$ cannot replace $\beta 2$ function (Rudolph et al., 1987, Michiels et al., 1987). The $\beta 1$ axonemes were much shorter and lacked the central pair microtubules (Raff et al., 2000).

The ability to make a functional axoneme must lie in the 25 different amino acids (Figure 7). Fourteen of the 25 differing amino acids between $\beta 1$ and $\beta 2$ were changed to $\beta 2$ identity in the $\beta 1$ gene (Nielsen et al., 2001). These changes included amino acids 55-57, 349, and 431-446. 55-57 and 349 are located in the internal variable region (IVR) and are involved in lateral contacts between the protofilaments (Nogales et al., 1998; Nogales et al., 1999). The carboxy terminus (431-446) is the site of most post-translational modifications (Rosenbaum, 2000) and also contains the axoneme motif. The axoneme motif is the two amino acids (EG at 433-434 followed by three acidic residues) required to make the central pair (Raff et al., 1997, Nielsen et al., 2001). The majority of these changes had an additive effect and increased $\beta 1$'s ability to make a motile axoneme by making it longer. Amino acids 55-57 actually had the opposite effect (Nielsen et al., 2001). They decreased $\beta 1$'s ability to make a functional axoneme. These axonemes were again much shorter. This decrease in ability suggests that amino acids 55-57 need $\beta 2$ -specific residues to properly function and are therefore interacting synergistically with other $\beta 2$ specific amino acids. There must be other $\beta 2$ amino acids required to complete a key interaction with 55-57.

One candidate amino acid for this synergistic relationship is residue 29. When looking at the 3D structure of the folded protein, amino acid 29 lies in the same

loop as 55-57, and is in direct contact with residues 55-57 (Figure 8). 29 is also unique in amino acid identity, only $\beta 2$ has a cysteine at 29 of all the β -tubulins in Genbank. This contact, and their unique identity in the evolutionary history of tubulins suggest they may have co-evolved in $\beta 2$, resulting in their synergistic function. To test this hypothesis amino acid 29 was changed from $\beta 1$ identity to $\beta 2$ identity in the $\beta 1$ gene using site directed mutagenesis, and transgenic flies were constructed with this new chimeric $\beta 1$ - $\beta 2$ protein (called TGARC) to determine if aa29 completes this synergism. We find that in fact, it largely does.

Methods

Site-Directed Mutagenesis Vector

The plasmid vector pBlueScript (stratagene) containing the $\beta 1$ - $\beta 2$ chimeric tubulin gene insert consisting of the $\beta 1$ gene with $\beta 2$ identity at amino acid codons 55-57, 349, and 431-446 (TGAR, Nielsen et al. 2001) was used as a template for site-directed mutagenesis. Following introduction of $\beta 2$ codon 29, the chimeric gene is referred to as TGARC (Figure 10).

Transformation Vector

The plasmid vector, pCaSpeRIV is a 7855 base-pair non-autonomous P-element vector (Figure 9). It contains an *EcoRI* site into which our TGARC chimeric gene is cloned. The *EcoRI* sites are flanked on both 5' and 3' sides by $\beta 2$ regulatory sequence sufficient to ensure the chimeric gene is expressed in the same place, time, and level as endogenous $\beta 2$ (Hoyle and Raff 1990). It also contains a wildtype copy of the white gene to allow visualization in transformed flies.

Site-Directed Mutagenesis

Site directed mutagenesis was used to change the identity of amino acid 29 from $\beta 1$ Gly to $\beta 2$ Cys in the pBluescript-TGAR vector. A mutagenic primer, (Figure 11) was generated to alter $\beta 1$ glycine to $\beta 2$ cysteine identity using a PCR approach

(Quick change site –directed mutagenesis kit, Stratgene.) The 50 μ L reaction consists of 5 ng double stranded template DNA, 50 ng each of forward and reverse mutagenic primers, 10x reaction buffer [100 mM KCl, 100 mM(NH₄)₂SO₄, 200 mM Tris-HCl (pH 8.8), 20 mM MgSO₄, 1% Triton® X-100, and 1 mg/ml nuclease-free bovine serum albumin (BSA)] dNTP's, quik solution [Stratagene] and water) was allowed to run for 18 cycles, during which the primer is incorporated into newly synthesized DNA. After the conclusion of PCR, the DNA was treated with 1 μ L of the endonuclease DpnI, to remove the parental DNA, which unlike newly synthesized sequence is dam methylated, and therefore targeted by the DpnI enzyme. This leaves only mutagenized plasmid remaining for transformation into competent *E. coli* cells.

Transformation

The newly synthesized plasmid DNA (TGARC) was transformed into *E. coli* competent cells (Stratagene). Cells were thawed on ice and 45 μ L of the cells were aliquoted into a pre-chilled Falcon® 2059 polypropylene tube. 2 μ L of β -mercaptoethanol mix was added and the tube was incubated on ice for 10 minutes, swirling every 2 minutes to mix. After 10 minutes, 2 μ L of the Dpn-I treated DNA was added. The tube was swirled gently to mix and then incubated on ice for 30 minutes. The tube was heat-pulsed in a 42°C water bath for exactly 30 seconds and placed on ice for two minutes. Next, 500 μ L of pre-heated (42°C) NZY⁺ broth was added and the tube was incubated at 37°C for 1 hour with shaking at 225-250 rpm. 100 μ L and 200 μ L of the newly transformed cells were plated on LB ampicillin agar

plates and placed into 37°C incubator overnight. After 24h, individual colonies were cultured overnight in LB ampicillin media for plasmid preparation.

DNA Isolation

Plasmid DNA was isolated using the QIAGEN plasmid purification system. The 50mL culture was centrifuged at 6000-x g for 15 minutes at 4° C and the supernatant was removed. The pellet was resuspended in 4mL of buffer P1 and vortexed until completely resuspended. Four ml of lysis buffer P2 was added and mixed gently by inversion and allowed to incubate at room temperature for 5 minutes. Next, 4 mL of chilled neutralizing buffer P3 was added, mixed gently by inversion and incubated on ice for 15 minutes. After 15 minutes the mixture was centrifuged at 20,000-x g for 30 minutes at 4° C and the supernatant was removed and placed into a new tube. The supernatant was precipitated using 1/10 the volume of 3M sodium acetate and 2x volume of ethanol. The mixture was then placed in -20° C overnight. The next day the solution was centrifuged for 30 minutes at 20,000 x g 4° C to pellet the DNA. The supernatant was removed and the pellet was resuspended in 100µl of water.

Purification and Ligation into CaSpeRIV P-element transformation vector

The TGARC chimeric gene was subjected to restriction endonuclease digestion using *EcoRI* endonuclease (50µL DNA, 4µl *EcoRI* enzyme and 6µl 10x buffer [New England BioLabs]) to extract it from the vector. The restriction digest

reaction was size fractionated by agarose gel electrophoresis (1% agarose, 1X buffer TAE at 100 milliamps for one hour), stained with ethidium bromide and visualized under UV light. The 1.7 Kb band corresponding to the $\beta 1$ - $\beta 2$ chimeric gene (TGARC) was excised out of the gel (Figure 12).

DNA was eluted from the gel using the QiaexII gel extraction kit. The excised band was weighed and 3 volumes of buffer QX1 was added to the gel slice. Next, 10 μ l of QIAEX II DNA-binding bead suspension was added and the tube was incubated at 50°C for 10 minutes, vortexing every 2 minutes to keep the QIAEX II in suspension. The sample was centrifuged for 30 seconds and the supernatant was removed. The remaining pellet was washed with 500 μ l of buffer QX1 and washed twice with 500 μ l buffer PE. The pellet was allowed to air dry 10-15 minutes until the pellet turned white and 20 μ l of water was added to elute the DNA.

CaSpeR IV vector was cut with the EcoRI restriction endonuclease and then treated with Calf-Intestinal Phosphatase (CIP) (20 μ l CaSpeRIV DNA in water, 2.2 μ l 1xNEB buffer, .4 μ l CIP; incubate at 37° C for one hour). CIP is an enzyme that removes the phosphates from the 5' ends to prevent the vector from religating back on itself. The TGARC DNA was ligated into the CIP treated CaSpeR IV vector (6 μ l TGARC DNA, 2 μ l CaSpeRIV DNA, 1 μ l 10x buffer, 1 μ l T4 DNA ligase, 4° C overnight). Proper orientation of the ligated insert relative to the regulatory regions

was confirmed by DNA sequencing (Northwoods DNA). CaSpeRIV-TGARC DNA was transformed into competent cells and isolated using the QIAGEN plasmid purification system (Figure 13). The pellet was resuspended in 100 μ l of fly injection buffer (5mM KCl and 0.1mM K₂HPO₄, pH 6.8), and quantified by gel electrophoresis.

Injection

Virgin w¹¹¹⁸ females and Δ 2-3 males (Table 1) are placed into a glass jar with media on top and allowed to mate and lay eggs for 45 minutes. After 45 minutes the media dish was removed and replaced with a fresh one and the newly fertilized embryos were removed from the media and placed on a slide. During this time the embryos are undergoing early embryogenesis and the cell is a syncytium, meaning multinucleated. Around nuclear division 9, a few nuclei migrate to the posterior end of the embryo and become enclosed by the plasma membrane. These are the pole cells that give rise to the gametes of the adult. After nuclear division 13, the embryo begins to cellularize and the plasma membrane closes around each nucleus forming the cellular blastoderm. This occurs approximately 90 minutes after fertilization, therefore it is important to ensure the injection occurs before this cellularization occurs to ensure the DNA can be taken up into the future germ cell nuclei.

The embryos were lined up next to each other, posterior side facing the end of the slide. Once lined up, halocarbon oil was laid overtop of the eggs to prevent drying during the injection procedure. A fine needle pulled from glass capillary tubes containing approximately 1 μ l of DNA, plus a small amount of green food dye to

visualize the injected DNA was inserted into a micromanipulator and pressure from a nitrogen tank was used to push a small amount of DNA into the embryo. Rarely, the DNA integrates into the germ cell genome where it will be expressed in the next generation.

Once injected, the embryos were placed into a fresh media dish and development is allowed to continue. After eclosion, crosses are performed to screen for the flies that contain the insert.

Genetic crosses

A number of chromosomes with dominant phenotypic markers were used in crosses to generate flies to determine which of the 3 fly chromosomes (X or Y, 2, and 3 – the 4th chromosomes are miniscule, and ignored in this procedure) the P-element landed in, and to test the function of TGARC (Table 1).

Embryo genotype:

P: w1118/w1118; +/+; +/+ **X** w1118/y; +/+; $\Delta 2-3/\Delta 2-3$

F1: w1118/w1118; +/+; $\Delta 2-3/+$

w1118 flies contain a mutant copy of the white gene and have white eyes (referred to as w from this point on). $\Delta 2-3$ is the source of the active transposase, a protein that binds to and inserts the P-element into the flies' genome. The P- element inserts itself randomly into the germline of this embryo, such that this flies progeny will have red eyes if the plasmid has been taken up. The P-element can only be inserted into germ

cells. Repression of P-element transposition in somatic cells occurs through RNA processing. A 97-kD protein binds to exon 2, thereby preventing the 2-3 intron from being spliced.

Identifying a P-element insert:

w/w; +/+; $\Delta 2-3/+$ X w/y; +/+; TM3/CXD

The TM3/CXD fly has two balancer third chromosomes; each contains dominant phenotypic markers that allow easy identification. TM3 flies have short, stubby thoracic bristles, and CXD flies have reduced bristle number and wings that extend perpendicular to the body axis of the fly. Flies with the P-element insert are transformed by its copy of the w⁺ gene have red eyes, due to rescue of the w⁻/w⁻ phenotype from the wildtype copy of the white gene contained on the P-element.

Determining the location of the insert:

P: P/y; +/+; +/TM3 X w/w; +/+; TM3/CXD

F1: P/w; +/+; +/TM3
y/w +/CXD
TM3/CXD

To explain how the location of the insert is found, it is useful to assume a location, in this example the X chromosome, and then show how this location can be verified. To begin, a red-eyed male progeny from the previous cross is mated to a white-eyed female with TM3/CXD occupying the third chromosomes. The F1 progeny of the cross are then examined for TM3 and CXD specific phenotypic characteristics. If one

of the progeny contains both TM3 and CXD and [P], the insert cannot be on the third chromosome because it is occupied by the balancers. If +/TM3 or +/CXD flies are found with [P], but a TM3/CXD [P] fly is never found, then the insert is on the third chromosome. If the insert is located on the X chromosome, then a red-eyed male will never be seen. This is because the male parent can only donate the insert [P] or the y, never both. If none of these conditions hold, then by elimination, [P] must be located on the second chromosome (Table 2).

Generating a fly with the insert as its only source of $\beta 2$:

P1: P/w; +/+; +/TM3 X w/y; +/+; K/TM3 (T and TM3 are the same balancer)

F1: P/w; +/+; K/T or P/y; +/+; K/T

P/w; +/+; K/T X P/y; +/+; K/T

F2: P/y; +/+; K/K

In order to determine the effects of the chimeric gene on spermatogenesis, the chimeric gene needs to be the only source of β -tubulin in the post-mitotic male germline. To create this fly, a red-eyed female with +/TM3 was mated to a white-eyed male with K/T on the third chromosome. The K is a $\beta 2$ null mutation with two recessive markers flanking the $\beta 2$ null mutation, radius incompletus (ri) and ebony (e). This fly will have a black body when present with the balancer TM3, which also has recessive ebony and radius incompletus alleles. T has the same stubby phenotype as TM3. Male and female progeny with the insert (P) and K/T are then mated to each

other to produce a male with two copies of $\beta 2$ null and one copy of the mutated form of $\beta 2$ (TGARC) as it's only source of $\beta 2$.

Determining fertility:

P: P/y; +/+; K/K X +/+; +/+; +/+

To determine if TGARC can support motile, fertile sperm, male flies with [P] as their only source of $\beta 2$ tubulin were mated to wildtype females, and the number of progeny produced in the cross were counted (N=0).

Analysis of the sperm produced by P/Y; +/+; K/K:

Sperm morphology in TGARC- $\beta 2$ null flies was visualized in orcein-stained testes.

Virgin males containing TGARC as their sole source of beta tubulin in the post-mitotic testis were isolated and dissected in TB buffer to remove their testes. Once removed, the testes were placed in 1% aceto-orcein stain for 1 minute, then placed on a subbed glass slide containing one drop of 60% acetic acid and a coverslip with one drop of 45% lacto-aceto orcein is placed on top. The coverslip was sealed to the slide with clear nail polish. The fixed testes were examined under the microscope.

Results

In order to examine if changing amino acid 29 from $\beta 1$ to $\beta 2$ identity restored the synergism lost when amino acids 55-57 are $\beta 2$, the chimeric $\beta 1$ - $\beta 2$ gene (TGARC) was expressed in the male germline. Transgenics containing the integrated chimeric gene in their genomes were examined in a number of different ways. They were first mated to virgin w1118 white-eyed females to test for fertility. Axoneme morphology was analyzed from testis stained with lacto-aceto orcein using phase contrast microscopy. Data show that amino acid Cys29 does participate in a synergism with 55-57 but does not fully rescue infertility caused by expressing chimeric forms of $\beta 1$ in place of $\beta 2$ tubulin in the male germline.

Transformants.

Two of the pair matings between embryos that survived the TGARC P-element injection procedure and w1118 flies resulted in red-eyed progeny, indicating such progeny were transformed by the P-element containing TGARC. These were two independent insertions, and in both cases subsequent crosses determined that the P-element was located on the X chromosome (Table 2). P-element insertions on the X are common. A 2nd chromosome insertion is preferable; because $\beta 2$ is located on chromosome 3, 2nd chromosome inserts allow testing of homozygote TGARC flies in homozygote $\beta 2$ null backgrounds. But the X-insertion does allow testing a hemizygous TGARC fly in a $\beta 2$ null background, and as $\beta 2$ +/ $\beta 2$ null flies are fertile, a single dose of our TGARC construct is sufficient to determine if it can generate fertile sperm.

We generated a “dump” stock of TGARC/TGARC; +/+; $\beta 2$ null/TM3 flies. This stock produces male flies containing one copy of TGARC in a $\beta 2$ null/ $\beta 2$ null background for use in fertility and morphology assays.

Fertility

15 male flies with TGARC as their only source of $\beta 2$ tubulin were then mated to virgin, white-eyed females to determine if TGARC restored male fertility. No progeny were produced in any of these crosses, thus indicating that TGARC is not able to restore fertility. However, further testing is currently being done to examine the effects of multiple copies of TGARC in different ratios to endogenous $\beta 2$ and $\beta 2$ null. Previous research has shown that all chimeric $\beta 1$ - $\beta 2$ genes, when expressed in a 1:1 ratio with endogenous $\beta 2$, can produce a motile axoneme. But when the ratio of the chimeric gene is 2:1 with endogenous $\beta 2$, the axoneme function is lost (Nielsen et al., 2001).

Morphology

The $\beta 1$ tubulin supports beta tubulin function in pre-meiotic germ cells (Kemphues et al., 1982). In the male germ line it is responsible for the singlet tubules of the mitotic spindle and cytoskeleton and tubules with the shared wall in the centriole (Hoyle and Raff, 1990). $\beta 2$ is first synthesized in mature primary spermatocytes just before meiosis and is expressed in the post-mitotic germ cells of the testis (Kemphues et al., 1982). It is required for all microtubule based functions

in the cell including the assembly of the meiotic spindles, axonemes and two classes of cytoplasmic microtubules (Kemphues et al., 1982) used in spermhead shaping, spermhead alignment, and elongation of the mitochondrial derivative. TGARC's ability to support these processes (except elongation of the mitochondrial derivative) was assessed using phase contrast microscopy on orecein-stained testes (Figure 14).

Meiosis

The presence of the normal complement of 64 sperm per cyst indicates TGARC supported functional meiotic spindles. The cyst, which consists of two somatic stem cells (cyst progenitor cells) surrounding a gonialblast, (germ line stem cell) is the basic unit of spermatogenic differentiation. The gonialblast undergoes four synchronous mitotic divisions which result in sixteen spermatogonia enclosed by two somatic cyst cells (which never divide). After the fourth round of division, the sixteen interconnected spermatogonia enter into the meiotic program where they enter prophase. Here they grow to 25x their volume and undergo extensive gene expression. After this, most transcription is shut down and the 16 primary spermatocytes undergo meiosis I and II. This results in 64 interconnected haploid spermatids in a cyst (Fuller 1998) (Figure 15).

Spermhead alignment and Spermhead shaping

Spermhead alignment results from microtubule-based interactions between the spermheads of the developing cyst and the cyst wall (Hoyle and Raff, 1990) and spermhead shaping from cytoskeletal microtubule arrays. The spermheads are

properly shaped and aligned, both of which are microtubule-based processes, indicating TGARC is able to support these singlet-microtubule-based aspects of spermatogenesis. This is compared to $\beta 2$ null/ $\beta 2$ null (Figure 16). In the absence of $\beta 2$, the spermatids do not elongate and the cysts remain round.

Axoneme morphogenesis

The sperm generated by five TGARC males were analyzed using phase contrast microscopy. The testes were dissected and stained with lacto-aceto orcein and 1% aceto orcein. Figure 14 shows the sperm from these are much longer (~ 1mm) than sperm generated with the most $\beta 2$ -like previously tested chimeric $\beta 1$ - $\beta 2$ tubulin (TGAR - ~ .01mm), indicating the single amino acid change at Cys29 restored axoneme-generating function to the TGAR protein. Although these sperm were longer, they also showed defects, breaks along the axoneme, and were not motile. This confirms that although TGARC seems to restore the synergism with 55-57, it is still not enough to generate a functional axoneme.

Discussion

The $\beta 1$ - $\beta 2$ tubulin chimeric protein containing Cys 29 did not rescue infertility in male *Drosophila melanogaster*. However, it did in part rescue the deleterious effects that amino acids Thr55 and Ala57 had on the length of the axoneme, indicating that Cys29 does participate in a $\beta 2$ -specific synergism with amino acids Thr55 and Ala57. Examination of the testes using aceto-orcein staining indicates TGARC generates sperm with much longer axonemes than supported by TGAR, and in fact much longer than any previously tested chimeric $\beta 1$ - $\beta 2$ tubulin (Nielsen et al. 2001).

Why does TGARC rescue only in part? This indicates that there are additional $\beta 2$ -specific amino acids required to make the axoneme functional. These unknown amino acids may also play a role in the synergistic relationship among Thr55, Ala57 and Cys29. Amino acids 29 and 55-57 are part of the Rossman fold, located in the nucleotide-binding domain of the protein (Nogales et al., 1998). This is the portion of the protein that binds the GTP nucleotide in the incoming tubulin dimer to the growing plus end of the microtubule. GTP hydrolysis occurs when the next subunit is added, and α binds to the exposed β tubulin. This changes the conformation of the microtubule, decreasing the strength of the lateral contacts between protofilaments. Amino acids 29 and 55-57 are specifically located in the S1-H2 loop. This loop is one of the most important involved in lateral contacts between protofilaments (Nogales et al., 1999). $\beta 2$ tubulin function is highly sensitive to the identity of residues in this loop, given that a single amino acid change has a 100x effect on axoneme length. Weakening of lateral protofilaments due to a lack of $\beta 2$ -

specific amino acids could explain the shortness of axonemes generated by chimeric $\beta 1$ - $\beta 2$ tubulins. With the incorrect conformation, the incoming subunit may not be able to bind, resulting in an exposed, GDP-beta tubulin cap and microtubule catastrophe.

Why is the $\beta 2$ protein so sensitive to its amino acid identity? There are three plausible explanations for this. The first one being that some proteins have a rigid structure/function relationship, meaning that only a specific structure can work. Stringency may not be intrinsic to the $\beta 2$ protein, but may result from having to form dimers with the major alpha tubulin isoform. The major insect alpha tubulin isoforms support all somatic microtubule function. In Dipterans, they also support the motile spermtail axoneme; in Lepidopterans the motile axoneme is supported by a testis-specific alpha and beta tubulin. The major alpha isoforms, both with and without testis function, are the most highly conserved insect tubulins, indicating that support of somatic, not testis function, constrains major alpha isoform evolution. This is evident when somatic and testis function are divided into distinct isoforms, as in Lepidopterans - the testis-specific alpha isoform evolves and the somatic alpha isoform does not. Moreover, the testis-specific beta isoform also evolves, and most rapidly at the inter- and intra- dimer interfaces where beta contacts alpha. This indicates that constraint on the major alpha tubulin extends through the Dipteran dimer to the testis-specific $\beta 2$ tubulin, constraining its evolution.

Stringency could also result from having to support such a long spermtail axoneme. *Drosophilid* spermtail axonemes are by far the longest in nature, and may require a highly specialized protein in their support. In a sense, if the axoneme is a

brick wall, it may be that a very square brick, such as $\beta 2$, is required to support a wall the height of the *Drosophila* axoneme. Small changes to the brick, for example substitution of $\beta 1$ -glycine rich identity into the $\beta 2$ S1-H2 loop as in the non-TGARC chimeric constructs, reduces the rigidity of the protein, which could lead to axoneme failure.

The third basis of stringency in $\beta 2$'s structure/function relationship to the axoneme relates to synergism. The amino acids involved in a synergism are interdependent of each other and must work together. However, they cannot evolve simultaneously, but must evolve one at a time. While some amino acids in the synergism are more tolerable to change and are able to maintain function, others such as Thr55 and Ala57, depend on specific amino acid identities in their contacts (ie. Cys29) to function properly. The order of evolution is thus important, the more tolerable amino acids must evolve first. This would slow down the rate of evolution because a change in an intolerable amino acid before that of a tolerable one would render the protein nonfunctional, therefore, only a subset of the possible evolutionary orders in which the synergism could evolve are actually allowable, because not all the pathways maintain a functional tubulin throughout.

Conclusions

Like the previous $\beta 1$ - $\beta 2$ chimeric genes, TGARC was able to support all microtubule-based structures except the axoneme. In fact, when compared to the other genes tested, TGARC functions better than the previous chimerics. For example when compared to TGAR, it can make axonemes 20x longer than those produced by TGAR (Nielsen et al., 2001), but it still cannot fully restore function. So despite being >99% similar to $\beta 2$, it cannot restore fertility to the fly. Because every combination of the remaining 13 differences between $\beta 1$ and $\beta 2$ cannot be tested, the final structure/function tests of the $\beta 2$ protein will focus on the 3 domains of the tubulin protein, rather than individual amino acids. Five mutagenic primers have been generated (Table 3) which will use a $\beta 1$ - $\beta 2$ construct consisting of the entire $\beta 1$ protein except for $\beta 2$ identity in the 3rd, carboxy terminus domain, as this is known to be fundamental to generating an axoneme (Nielsen et al. 2001). The entire first, nucleotide-binding domain will be mutated with the first 3 sets of primers, resulting in a protein that is $\beta 2$ - $\beta 1$ - $\beta 2$ over the three domains of the protein (nucleotide-binding, drug-binding, carboxy-terminus). A second construct, $\beta 1$ - $\beta 2$ - $\beta 2$, will be generated and their functions compared to determine if one or the other domains is the source of the remaining loss of function in the previously tested chimeric constructs. This will reveal if $\beta 2$'s functional specialization resides in an unusual nucleotide-binding property, or involves the structural core of the protein, the drug-binding domain.

LITERATURE CITED

1. Becker et al. 2000. The World of the Cell. Addison Wesley Longman, Inc., San Francisco, CA, pp 752-753.
2. Biernat, J., N. Gustke, G. Drewes, E. M. Mandelkow, and E. Mandelkow. 1993. Phosphorylation of Ser (262) Strongly Reduces Binding of Tau-Protein to Microtubules - Distinction between Phf-Like Immunoreactivity and Microtubule-Binding. *Neuron* 11(1): 153-163.
3. Brown, J.M., C. Marsala, R. Kosoy, and J. Gaertig. 1999. Kinesin-II is preferentially targeted to assembling cilia and is required for ciliogenesis and normal cytokinesis in *Tetrahymena*. *Mol. Biol. Cell* 10:3081-3096.
4. Cole, D.G., S.W. Chinn, K.P. Wedaman, K. Hall, T. Vuong, and J.M. Scholey. 1993. Novel heterotrimeric kinesin-related protein purified from sea urchin eggs. *Nature* 366:268-270.
5. Cole, D.G., D.R. Diener, A. Himmelblau, P.L. Beech, J.C. Fuster, and J.L. Rosenbaum. 1998. *Chlamydomonas* kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in *Caenorhabditis elegans* sensory neurons. *J. Cell Biol.* 141:993-1008.
6. Cole, D. G. 1999. Kinesin-II, coming and going. *J. Cell Biol.* 147(3): 463-465.

7. Charrasse, S., M. Schroeder, C. Gauthier-Rouviere, L. Cassimeris D. L. Gard and C. Larroque. 1996. The human ch-TOG protein is associated with mitotic spindles and is homologous to the *Xenopus* microtubule associated protein, XMAP215. *Mol. Biol. Cell* 7: 1292-1292.
8. Curmi, P. A., S. S. L. Andersen, S. Lachkar, O. Gavet, E. Karsenti, M. Knossow, and A. Sobel. 1997. The stathmin/tubulin interaction *in vitro*. *J. Biol. Chem.* 272(40): 25029-25036.
9. Desai, A. and T. J. Mitchison. 1997. Microtubule polymerization dynamics. *Annual Review of Cell and Developmental Biology* 13: 83-117.
10. Desai, A., Verma, S., Mitchison, T.J., and Walczak, C.E. 1999. Kin I kinesins are microtubule-destabilizing enzymes. *Cell* 96, 69-78.
11. Drechsel, D. N., A. A. Hyman, M. H. Cobb, and M. W. Kirschner. 1992. Modulation of the Dynamic Instability of Tubulin Assembly by the Microtubule-Associated Protein Tau. *Mol. Biol. Cell* 3(10): 1141-1154.
12. Drewes, G., A. Ebner, and E. M. Mandelkow. 1998. MAPs, MARKs, and microtubule dynamics. *TIBS* 23: 307-311.
13. Dutcher, S.K. 1995. Flagellar assembly in two hundred and fifty easy-to-follow steps. *Trends Genet.* 11: 398-404.
14. Fuller, M. 1993. Spermatogenesis. In The Development of *Drosophila melanogaster*, Volume 2. eds. M. Bate and A. Martinez Arias. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory pp. 71-147.

15. Gard, D. L. and M. W. Kirschner 1987. A Microtubule-Associated Protein from *Xenopus* Eggs That Specifically Promotes Assembly at the Plus-End. *J. Cell Biol.* 105(5): 2203-2215.
16. Gibbons, I. R. 1981. Cilia and flagella of eukaryotes. *J. Cell Biol.* 91:107-124.
17. Graur, D., and Li, W.H. 2000. Fundamentals of Molecular Evolution. Sinauer Associates, Inc., Sunderland, Massachusetts. p 104.
18. Han, Y. G., B. H. Kwok, and M. J. Kernan. 2003. Intraflagellar transport is required in *Drosophila* to differentiate sensory cilia but not sperm. *Current Biology* 13(19): 1679-1686.
19. Horio, T. and H. Hotani. 1986. Visualization of the Dynamic Instability of Individual Microtubules by Dark-Field Microscopy. *Nature* 321(6070): 605-607.
20. Hoyle, H. D. and E. C. Raff 1990. Two *Drosophila* Beta Tubulin Isoforms Are Not Functionally Equivalent. *J. Cell Biol.* 111(3): 1009-1026.
21. Huang, B., G. Piperno, and D. J. L. Luck. 1979. Paralyzed flagella mutants of *Chlamydomonas reinhardtii*. *J. Biol. Chem.* 254:3091-3099.
22. Huang, B., G. Piperno, Z. Romanis, and D. J. L. Luck. 1981. Radial spokes of *Chlamydomonas* flagella: genetic analysis of assembly and function. *J. Cell Biol.* 88:80-88.
23. Hutchens, J. A., H. D. Hoyle, F. R. Turner, and E. C. Raff. 1997. Structurally similar *Drosophila* alpha-tubulins are functionally distinct in vivo. *Mol. Biol. Cell* 8(3): 481-500.

24. Hyams J. S., Lloyd, C. W., eds. 1994. *Microtubules*. Wiley-Liss. New York. pp 439.
25. Jourdain, L., P. Curmi, A. Sobel, D. Pantaloni, and M. F. Carlier. 1997. Stathmin is a tubulin-sequestering protein which forms a ternary T2S complex with two tubulin molecules. *Biochemistry* 36: 10817-10821.
26. Kaltschmidt, B., K. H. Glätzer, F. Michiels, D. Leiss, and R. Renkawitz-Pohl. 1991. During *Drosophila* spermatogenesis $\beta 1$, $\beta 2$, and $\beta 3$ tubulin isotypes are cell-type specifically expressed but have the potential to coassemble into the axoneme of transgenic flies. *Eur. J. Cell Biol.* 54: 110-120.
27. Kozminski, K.G., K.A. Johnson, P. Forscher, and J.L. Rosenbaum. 1993. A motility in the eukaryotic flagellum unrelated to flagellar beating. *Proc. Natl. Acad. Sci. USA.* 90:5519-5523.
28. Kozminski, K.G., P.L. Beech, and J.L. Rosenbaum. 1995. The *Chlamydomonas* kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane. *J. Cell Biol.* 131:1517-1527.
29. Larsson, N., B. Segerman, B. Howell, K. Fridell, L. Cassimeris, and M. Gullberg. 1999. Op18/stathmin mediates multiple region-specific tubulin and microtubule regulating activities. *Mol. Biol. Cell* 10: 2178.
30. Lowe, J., H. Li, K. H. Downing, and E. Nogales. 2001. Refined structure of alpha beta-tubulin at 3.5 Å resolution. *J. Mol. Biol.* 313(5): 1045-1057.
31. Luck, D. 1984. Genetic and biochemical dissection of the eucaryotic flagellum. *J. Cell Biol.* 98(3): 789-794.

32. Mandelkow, E. M., E. Mandelkow, and R. A. Milligan. 1991. Microtubule Dynamics and Microtubule Caps - a Time-Resolved Cryoelectron Microscopy Study. *J. Cell Biol.* 114(5): 977-991.
33. Marszalek, J.R., P. Ruiz-Lozano, E. Roberts, K.R. Chien, and L.S. Goldstein. 1999. Situs inversus and embryonic ciliary morphogenesis defects in mouse mutants lacking the KIF3A subunit of kinesin-II. *Proc. Natl. Acad. Sci. USA* 96:5043-5048.
34. McNally, F. J. 1999. Microtubule dynamics: Controlling split ends. *Current Biology* 9(8): R274-R276.
35. Michiels, F., D. Falkenburg, A. M. Müller, U. Hinz, U. Otto, and R. Bellman. 1987. Testis-specific $\beta 2$ tubulins are identical in *Drosophila melanogaster* and *D. hydei* but differ from ubiquitous $\beta 1$ tubulin. *Chromosoma*. 95: 387-395.
36. Mitchison, T. and M. Kirschner 1984. Dynamic Instability of Microtubule Growth. *Nature* 312(5991): 237-242.
37. Morris, R.L., and J.M. Scholey. 1997. Heterotrimeric kinesin-II is required for the assembly of motile 9+2 ciliary axonemes on sea urchin embryos. *J. Cell Biol.* 138:1009-1022.
38. Nielsen, M. G., F. R. Turner, J. A. Hutchens, and E. C. Raff. 2001. Axoneme-specific beta-tubulin specialization: a conserved C- terminal motif specifies the central pair. *Current Biology* 11(7): 529-533.
39. Nielsen submitted

40. Nogales, E., S. G. Wolf and K. H. Downing. 1998. Structure of the alpha beta tubulin dimer by electron crystallography. *Nature* 391(6663): 199-203.
41. Nogales, E., M. Whittaker, R. A. Milligan and K. H. Downing. 1999. High-resolution model of the microtubule. *Cell* 96(1): 79-88.
42. Nonaka, S., Y. Tanaka, Y. Okada, S. Takeda, A. Harada, Y. Kanai, M. Kido, and N. Hirokawa. 1998. Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell* 95:829-837.
43. Oakley, B. R., Oakley, C. E., Yoon, Y., Jung, M. K. 1990. Gamma-tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell* 61(7): 1289-1301.
44. O'Farrell, P. H. 1975. High-resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250: 4007-4021.
45. Pazour, G.J., B.L. Dickert, and G.B. Witman. 1999. The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagellar assembly. *J. Cell Biol.* 144:473-481.
46. Pfister, K. K., R. B. Fay, and G. B. Witman. 1982. Purification and polypeptide composition of dynein ATPases from *Chlamydomonas* flagella. *Cell Motility* 2:525-547.
47. Piperno, G., B. Huang, and D. J. L. Luck. 1977. Two-dimensional analysis of flagellar proteins from wild-type and paralyzed mutants of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* 74:1600-1604.

48. Piperno, G., and D. J. L. Luck. 1979. Axonemal adenosine triphosphatases from flagella of *Chlamydomonas reinhardtii*: purification of two dyneins. *J. Biol. Chem.* 254:3084-3090.
49. Piperno, G., and D. J. L. Luck. 1981. Inner arm dyneins from flagella of *Chlamydomonas reinhardtii*. *Cell* 27:331-340.
50. Pitnick, S., Spicer, G.S., and Markow, T.A. 1995. How long is a giant sperm? *Nature* 375, 109.
51. Porter, M., K. Bower, J. Knott, P. Byrd, and W. Dentler. 1999. Cytoplasmic dynein heavy chain 1b is required for flagellar assembly in *Chlamydomonas*. *Mol. Biol. Cell* 10:693-712.
52. Pryer, N. K., R. A. Walker, V. P. Skeen, B. D. Bourns, M. F. Soboeiro, and E. D. Salmon. 1992. Brain Microtubule-Associated Proteins Modulate Microtubule Dynamic Instability *In vitro* - Real-Time Observations Using Video Microscopy. *Journal of Cell Science* 103: 965-976.
53. Raff, E. C. 1994. The role of multiple tubulin isoforms in cellular microtubule function. In J.S. Hyams and C.W. Lloyd (eds.). *Microtubules*. John Wiley & Sons, Inc., New York, pp 85-110.
54. Raff, E. C., J. D. Fackenthal, J. A. Hutchens, H. D. Hoyle, and F. R. Turner. 1997. Microtubule architecture specified by a beta-tubulin isoform. *Science* 275(5296): 70-73.
55. Raff, E. C., J. A. Hutchens, H. D. Hoyle, M. G. Nielsen, and F. R. Turner. 2000. Conserved axoneme symmetry altered by a component beta-tubulin. *Current Biology* 10(21): 1391-1394.

56. Rosenbaum, J. 2000. Cytoskeleton: Functions for tubulin modifications at last. *Current Biology* 10(21): R801-R803.
57. Rudolph, J. E., M. Kimble, H. D. Hoyle, M. A. Subler, and E. C. Raff. 1987. 3 *Drosophila* Beta-Tubulin Sequences - a Developmentally Regulated Isoform (Beta-3), the Testis-Specific Isoform (Beta- 2), and an Assembly-Defective Mutation of the Testis-Specific Isoform (β 2t8) Reveal Both an Ancient Divergence in Metazoan Isotypes and Structural Constraints for Beta-Tubulin Functions. *Molecular and Cellular Biology* 7(6): 2231-2242.
58. Signor, D., K.P. Wedaman, J.T. Orozco, N.D. Dwyer, C.I. Bargmann, L.S. Rose, and J.M. Scholey. 1999a. Role of CHE-3 dynein in retrograde transport of IFT motors and IFT raft particles along cilia, but not dendrites, in chemosensory neurons of living *C. elegans*. *J. Cell Biol.* 147:519-530
59. Signor, D., K.P. Wedaman, L.S. Rose, and J.M. Scholey. 1999b. Two heteromeric kinesin complexes in chemosensory neurons and sensory cilia of *Caenorhabditis elegans* *Mol. Biol. Cell* 10:345-360.
60. Takeda, S., Y. Yonekawa, Y. Tanaka, Y. Okada, S. Nonaka, and N. Hirokawa. 1999. Left-right asymmetry and kinesin superfamily protein KIF3A: new in-sights in determination of laterality and mesoderm induction by kif3A 2/2 mice analysis. *J. Cell Biol.* 145:825-836.
61. Bates, A. 1971. Cytodifferentiation during Spermatogenesis in *Drosophila* (Leiden, The Netherlands: Rijksuniversiteit).

62. Trinczek, B., J. Biernat, K. Baumann, E. M. Mandelkow, and E. Mandelkow. 1995. Domains of Tau-Protein, Differential Phosphorylation, and Dynamic Instability of Microtubules. *Mol. Biol. Cell* 6(12): 1887-1902.
63. Vasquez, R. J., D. L. Gard, and L. Cassimeris. 1994. Xmap from *Xenopus* Eggs Promotes Rapid Plus End Assembly of Microtubules and Rapid Microtubule Polymer Turnover. *J. Cell Biol.* 127(4): 985-993
64. Warner, F. D., and P. Satir. 1974. The structural basis of ciliary bend formation: radial spoke positional changes accompanying microtubule sliding. *J. Cell Biol.* 63:35-63.
65. Walczak, C.E., Mitchison, T.J., and Desai, A. 1996. XKCM1: a *Xenopus* kinesin-related protein that regulates microtubule dynamics during mitotic spindle assembly. *Cell* 84, 37-47.
66. Yamazaki, H., T. Nakata, Y. Okada, and N. Hirokawa. 1996. Cloning and characterization of KAP3: a novel kinesin superfamily-associated protein of KIF3A/3B. *Proc. Natl. Acad. Sci. USA.* 93:8443-8448
67. Zheng, Y. X., M. L. Wong, B. Alberts and T. Mitchison. 1995. Nucleation of Microtubule Assembly by a Gamma-Tubulin- Containing Ring Complex. *Nature* 378(6557): 578-583.

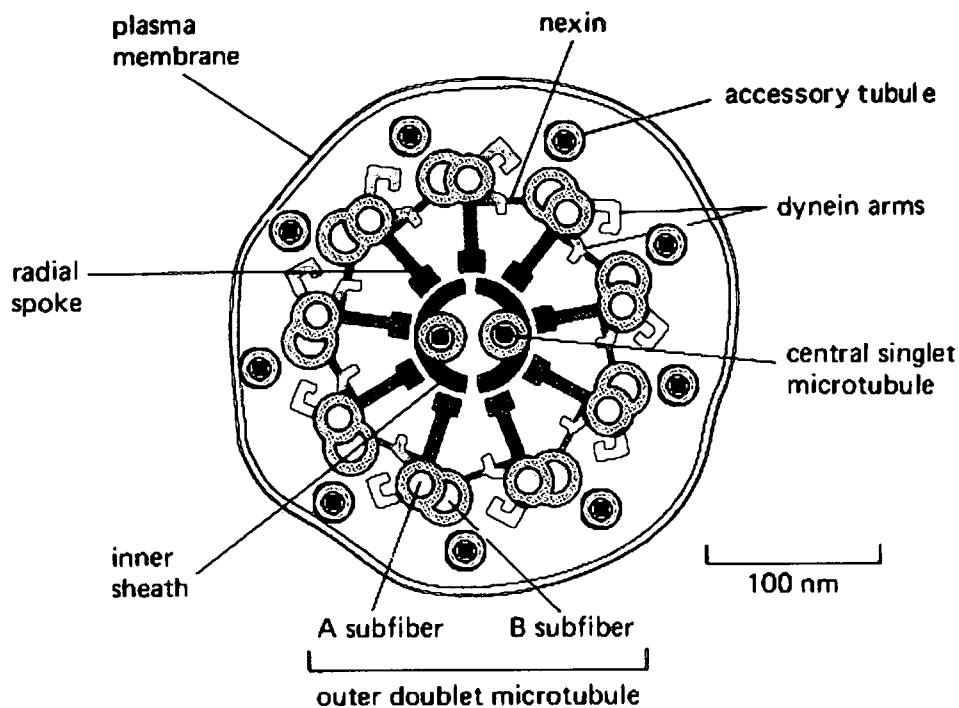


Figure 1: Schematic drawing of the axoneme with accessory structures. It contains the canonical 9+2 ultrastructure of nine doublet microtubules surrounding a central pair of singlet microtubules. It also has accessory tubules, inner and outer dynein arms and radial spokes. The dynein arms bind and release the adjacent doublet in an ATP-dependant manner to cause flagellar beating, while the radial spokes help regulate distal sliding in conjunction with the central pair.

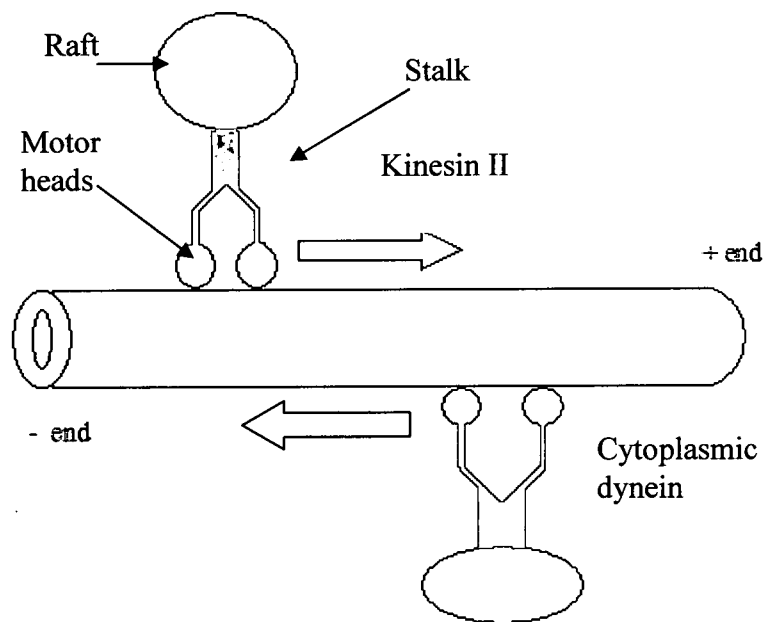


Figure 2: Intraflagellar Transport. Kinesin II is responsible for anterograde IFT towards the plus end of the microtubule, while cytoplasmic dynein controls retrograde IFT. The motor heads get their power from the hydrolysis of ATP to ADP + P_i .

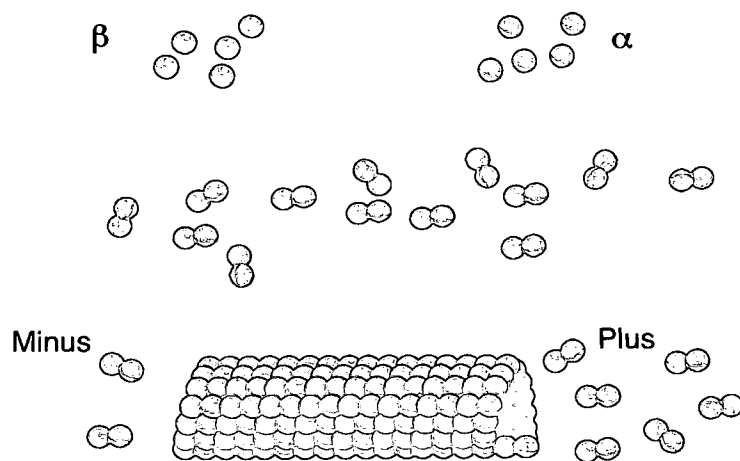


Figure 3: α and β monomers and heterodimers combine to form protofilaments. Thirteen protofilaments associate laterally to form the microtubule.

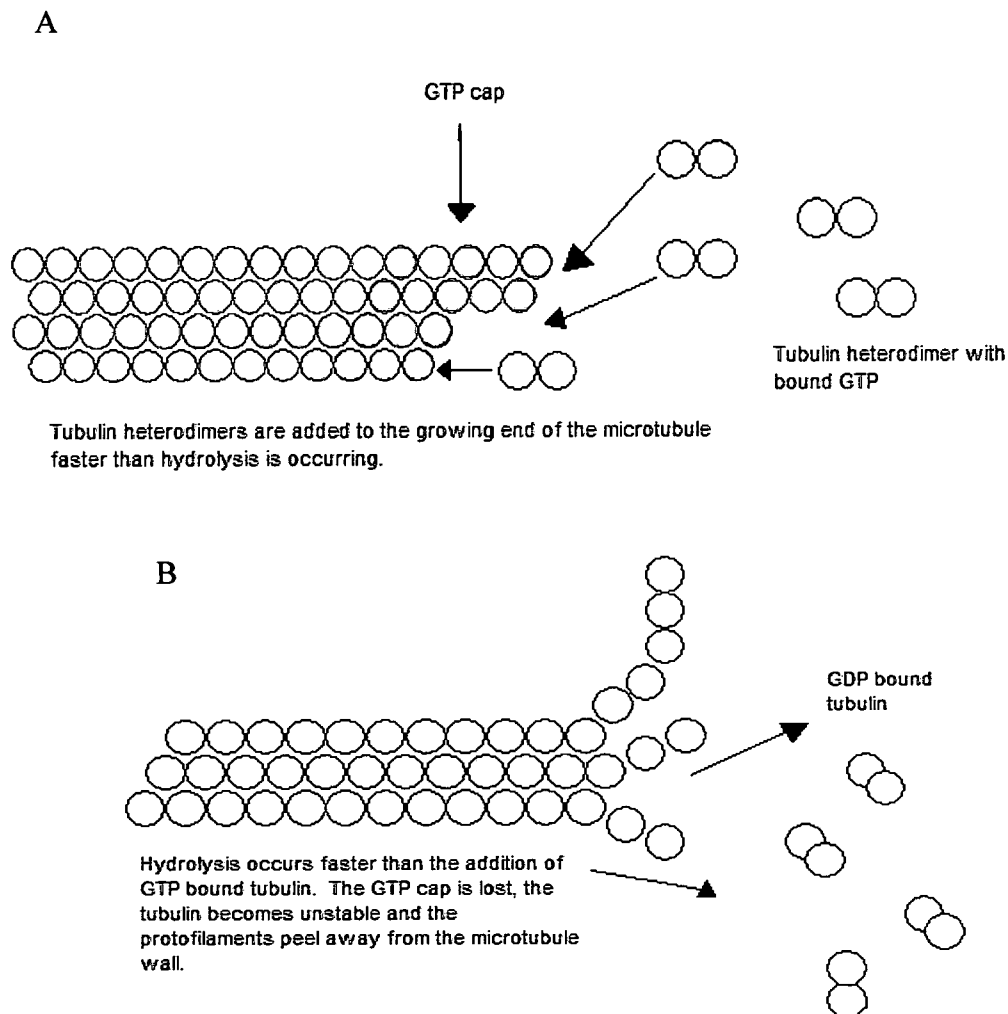


Figure 4: Dynamic instability. (A) Growing microtubule. GTP-bound tubulin is added to the plus end of the microtubule faster than hydrolysis can occur. This adds a GTP cap to the end and stabilizes it. (B) Shrinking microtubule. Hydrolysis occurs faster than the addition of the tubulin dimers. This results in the loss of the GTP cap and the microtubule becomes unstable. The protofilaments peel away and the GDP-bound tubulin returns to the cytosol to exchange its GDP for GTP. It is ready to be added to another growing microtubule.

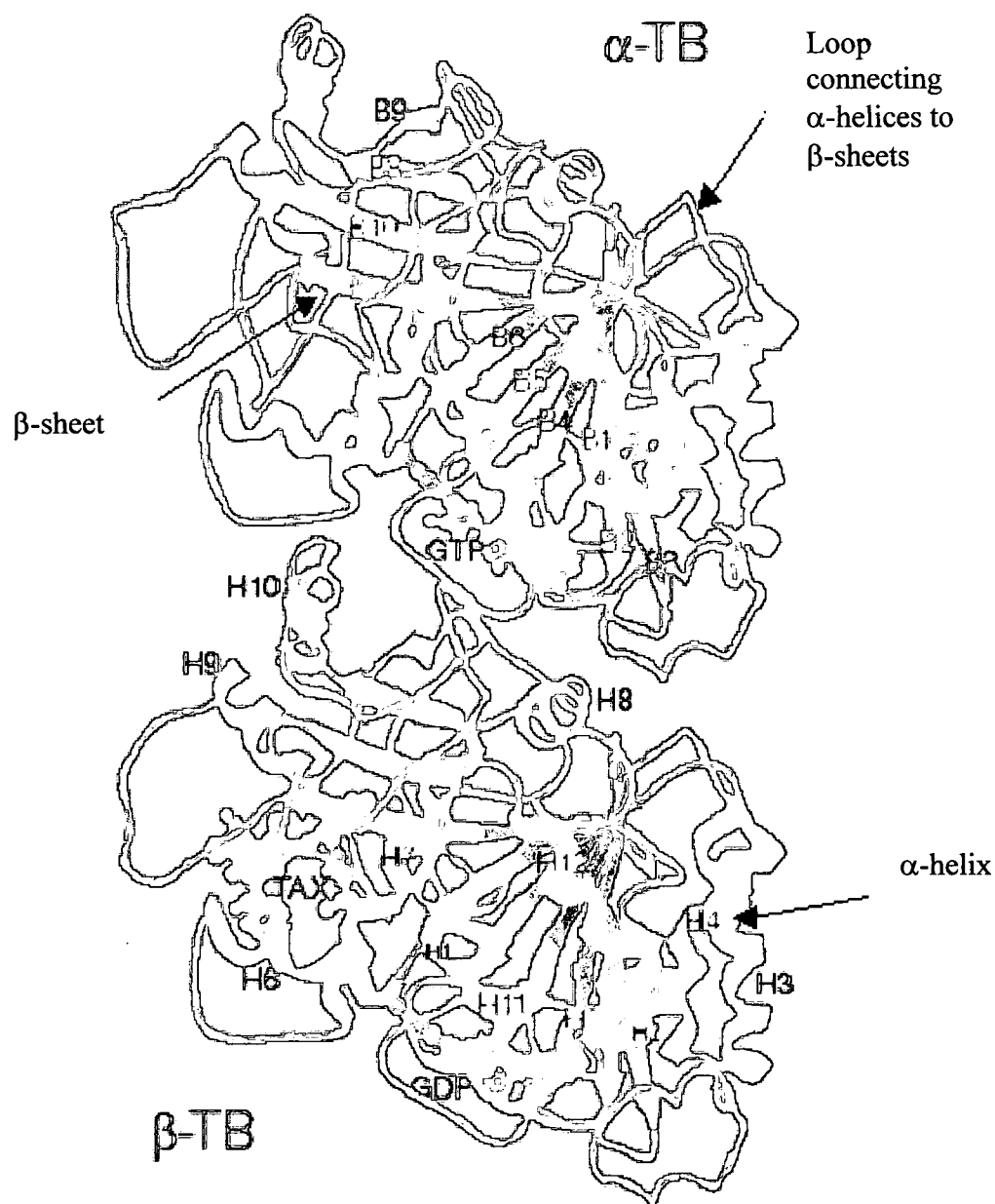


Figure 5: Ribbon diagram of α and β tubulin. The α -helices (H) and β -sheets (B) are labeled. The top shows α tubulin bound to GTP while the bottom shows β tubulin bound GDP and taxotere. This figure was produced using Advanced Visual; ribbon module from M. Carson and A. Shah. (Nogales et al., 1998).



Figure 6: Electron micrograph of an axoneme from a sterile male with two copies of $\beta 1$ in place of $\beta 2$. The central pair microtubules are missing but the morphology is otherwise normal. This section was taken within 2μ of the basal body of an early stage axoneme (Nielsen et al. 2001).

Beta 1 X Beta 2 Tubulin

beta1	MREIVHIQAGQCGNQIGAKFWEIISDEHGIDATGTY H GDSDLQLERINVYYNEASGGKYV
beta2	MREIVHIQAGQCGNQIG G KFWEVISDEHCIDATGTYYGDSDLQLERINVYYNEATGAKYV
beta1	PRAVLVDLEPGTMDSVRSG P FGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVV
beta2	PRA I LVDLEPGTMDSVRSG R FGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVV
beta1	RKEAESCDC L QGFQLTHSLGGGTGSGMG T LLISKIREEYPDRSMNTYSVVPSPKVSDTVV
beta2	RKESEGCDC L QGFQLTHSLGGGTGSGMG T LLISKIREEYPDRIMNTFSVVPSPKVSDTVV
beta1	EPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDLNHLVSLTMSGVTTCL
beta2	EPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDLNHLVSATMSGVTTCL
beta1	RFPGQLNADLRKLAVNMVFPRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMFDKNNMM
beta2	RFPGQLNADLRKLAVNMVFPRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMFDKNNMM
beta1	AACDPRHGRYLTVA A IFRGRMSMKEVDEQMLNIQNKNSS Y FVEWIPNNVKTAVCDIPPRG
beta2	AACDPRHGRYLTVA A IFRGRMSMKEVDEQMLNIQNKNSS F FVEWIPNNCKTAVCDIPPRG
beta1	LKMSATFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGE G MDMEFTEAESNMNDLVS
beta2	LKMSATFIGNSTAIQELFKRVSEQFTAMFRRKAFLHWYTGE G MDMEFTEAESNMNDLVS
beta1	EYQQYQEATADE D A E FE E E E Q A EV D EN
beta2	EYQQYQEATADE E GE F DE D EE G GGDE-

Figure 7: Comparison of the $\beta 1$ and $\beta 2$ amino acid sequence in *Drosophila melanogaster*. There are only 25 differences between the two, but $\beta 1$ can't replace $\beta 2$ to make a functional axoneme. The differences are highlighted and in bold.

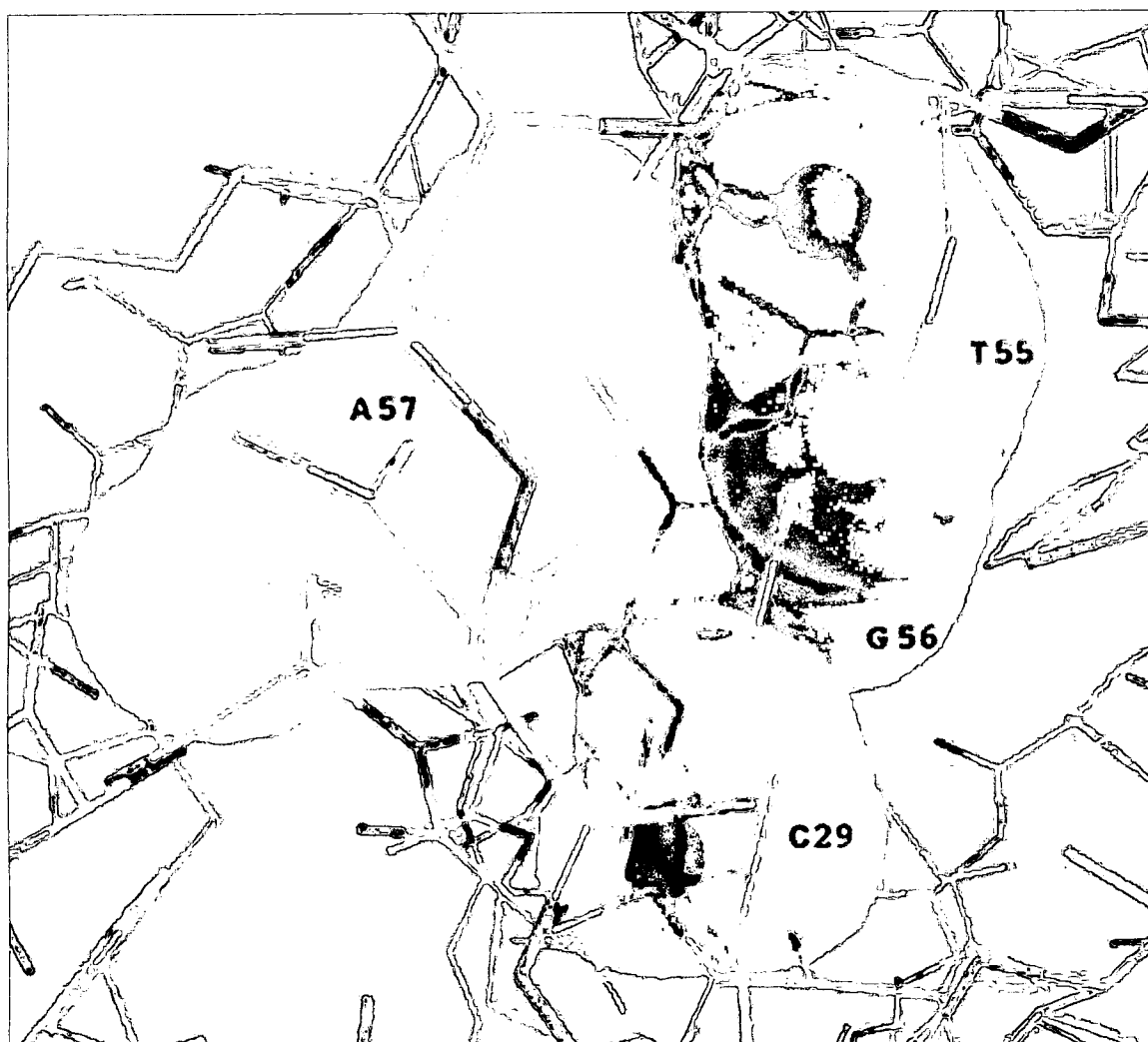


Figure 8: Three-dimensional model of amino acids 29 and 55-57 in $\beta 2$ tubulin. Cysteine 29 is green, Alanine 57 is yellow, Threonine 55 is purple and Glycine 56 is red. Cys 29 is a good candidate amino acid to restore the synergism with 55-57 that is lost when amino acids 55-57 are changed to $\beta 2$ identity. It is in direct contact with 55-57 in the folded protein.

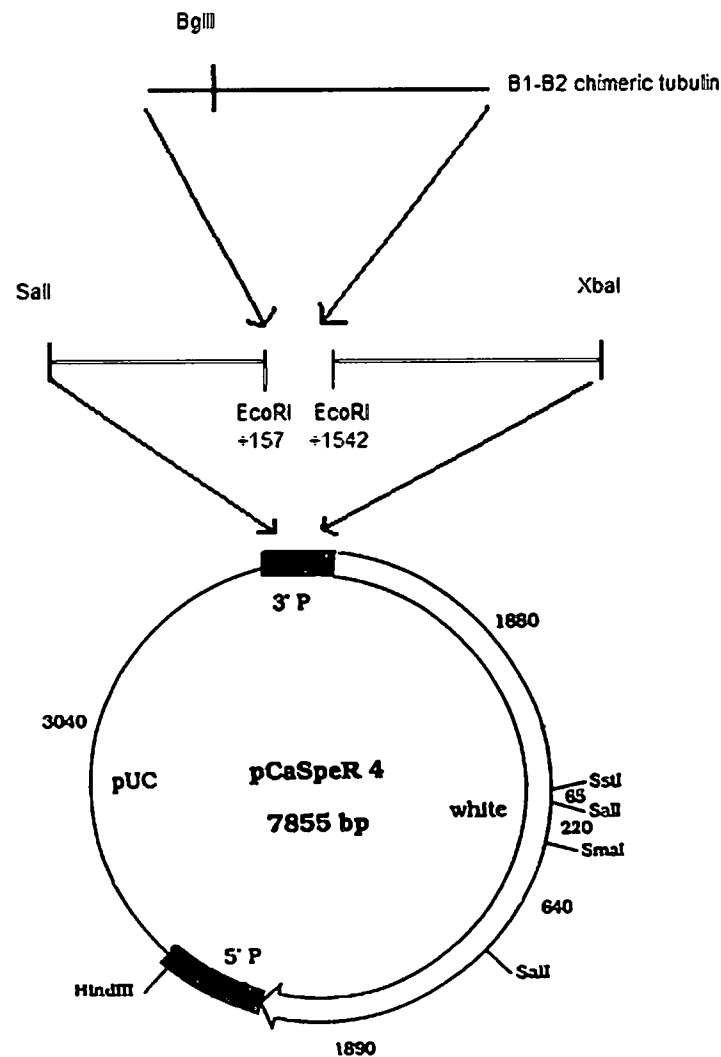


Figure 9: pCaSpeR IV vector used to insert the $\beta 1$ - $\beta 2$ chimeric gene into the *Drosophila* germline. It is a 7855 base pair P-element vector containing both the 5' and 3' P-elements necessary to integrate the chimeric gene into the fly's genome. It also contains a wildtype copy of the white gene that is used to indicate that the DNA has been integrated, and the 5' and 3' $\beta 2$ tubulin regulatory sequences to ensure that the chimeric gene is expressed exactly like the endogenous $\beta 2$. The chimeric gene is ligated into the vector via the *EcoRI* restriction endonuclease sites.

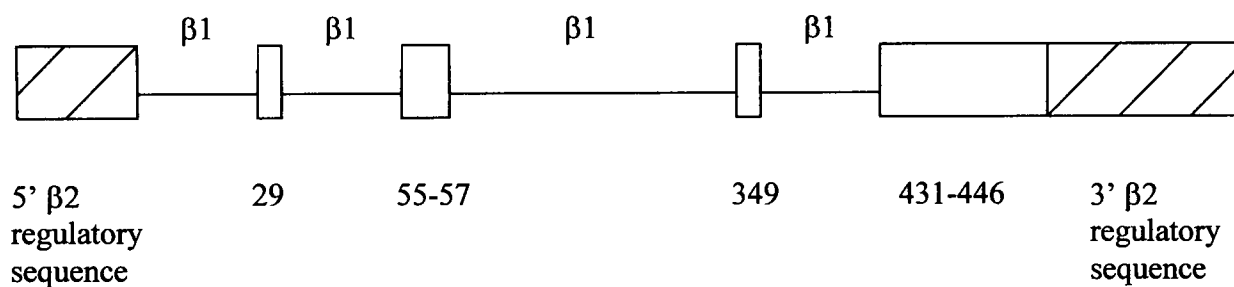


Figure 10: Diagram of the $\beta 1$ - $\beta 2$ tubulin chimeric gene TGARC. The striped boxes indicate the $\beta 2$ regulatory sequence used to ensure the chimeric gene is expressed exactly as the endogenous $\beta 2$. The line indicates the $\beta 1$ sequence and the filled boxes are $\beta 2$ identity at these specific residues.

Nucleotide	CC	GAT	GAG	CAT	TGC	ATC	GAT	GCC	ACC	GG
Amino acid	S	D	E	H	C	I	D	A	T	G

Figure 11: Mutagenic primer used to change amino acid 29 from $\beta 1$ identity to $\beta 2$ identity. $\beta 2$ sequence is in blue and $\beta 1$ is in black.

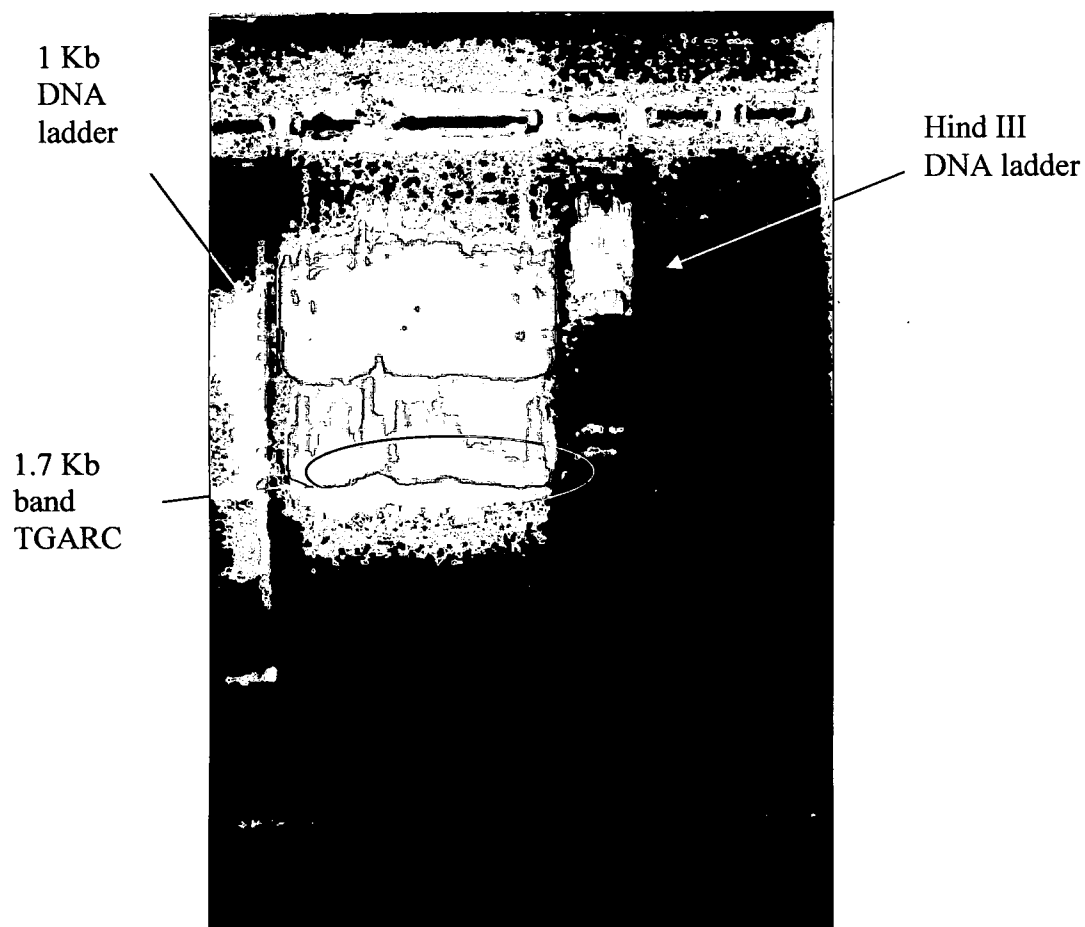


Figure 12: Photograph of 1% agarose gel electrophoresis stained with ethidium bromide. The blue circle indicates the 1.7 Kb band that corresponds with the $\beta 1$ - $\beta 2$ chimeric gene (TGARC). This band was excised out of the agarose and the DNA was eluted.

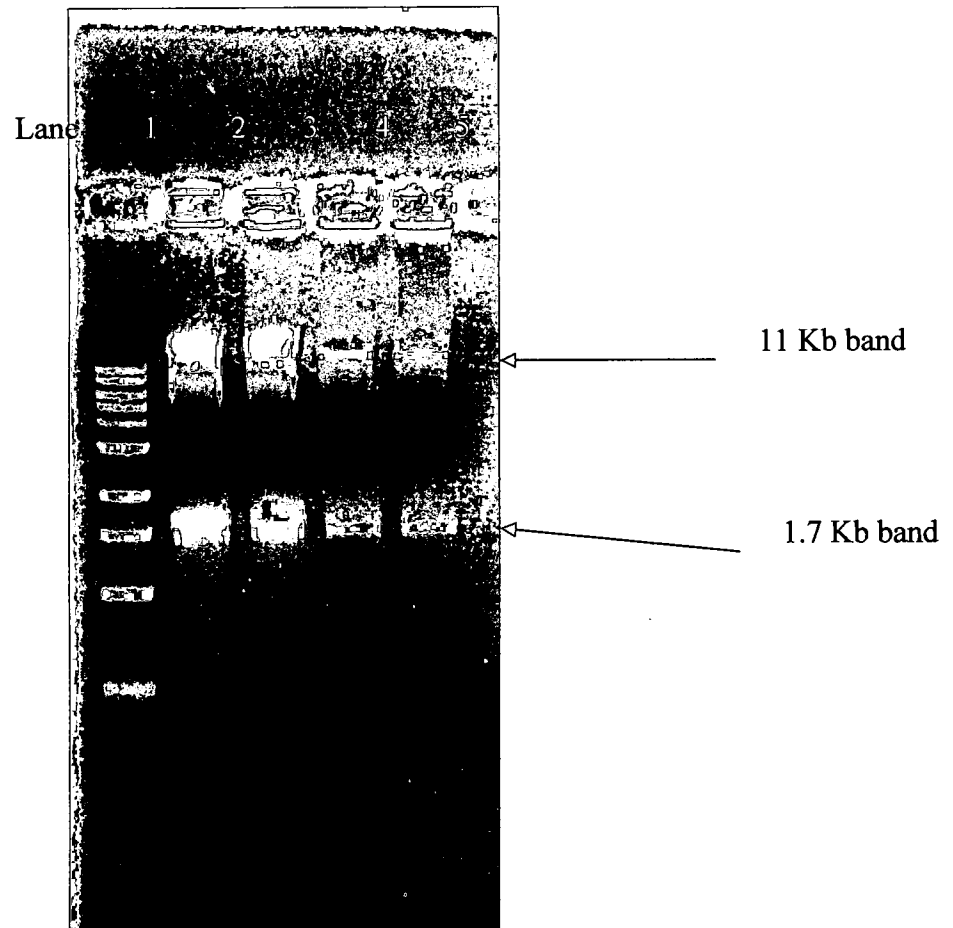


Figure 13: Photograph of 1% agarose gel electrophoresis stained with ethidium bromide. The pCaSper IV vector containing TGARC was digested using the *EcoRI* restriction endonuclease. Lane 1 represents the 1 Kb DNA ladder used for reference. Lanes 2 and 4 represent sample # 4, and lanes 3 and 5 represent sample #1. The presence of both the 1.7Kb and 11 Kb bands confirms that the chimeric $\beta 1$ - $\beta 2$ gene (TGARC) was successfully ligated into the pCaSpeR IV vector in each sample.

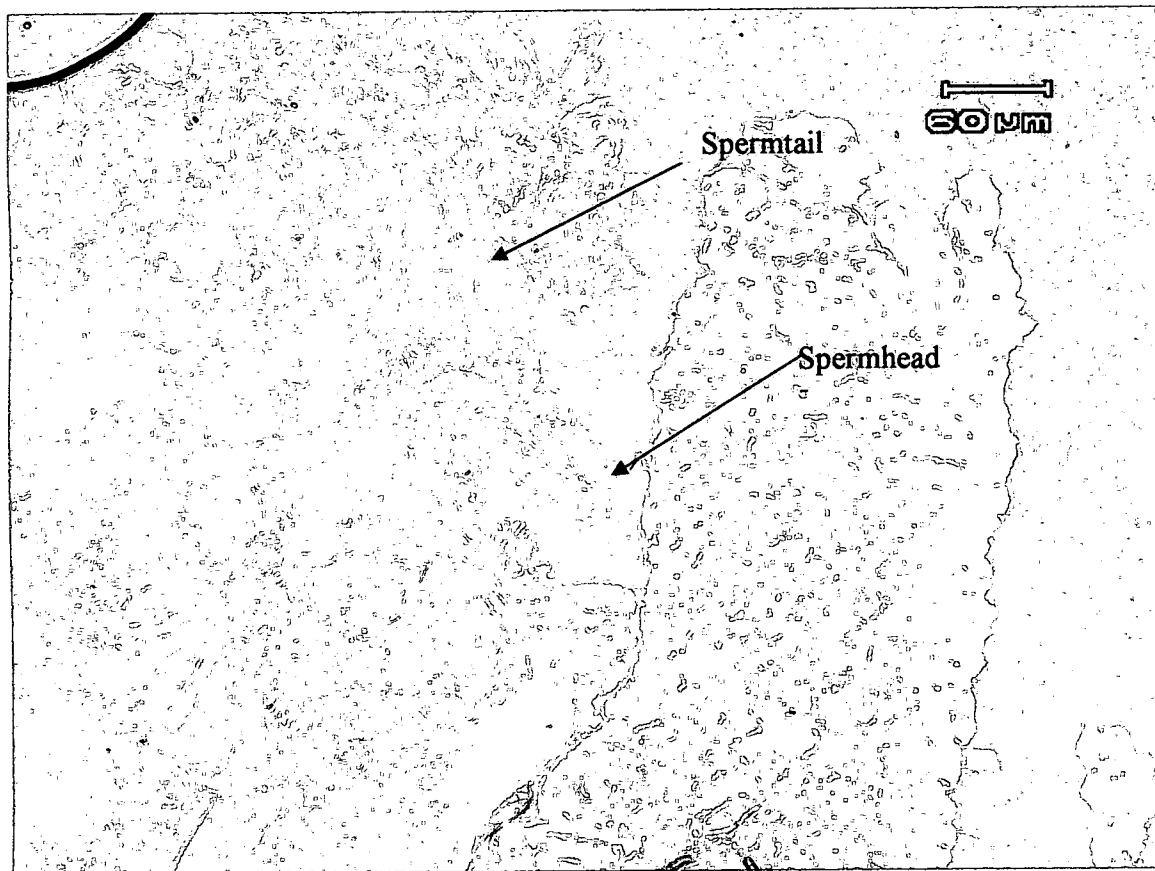


Figure 14: Elongating spermatids. Light micrograph viewed under phase contrast of elongating spermatids from male flies with one copy of the chimeric $\beta 1$ - $\beta 2$ gene, TGARC in a $\beta 2$ null/ $\beta 2$ null background. The spermtail are 20x longer (~ 1 mm) than those of the previous chimeric $\beta 1$ - $\beta 2$ gene, TGAR (~ 0.1 mm) (Nielsen et al., 2001). This indicates the single amino acid change at Cys29 restored axoneme-generating function to the TGAR protein. Scale bar represents $60\mu\text{m}$.

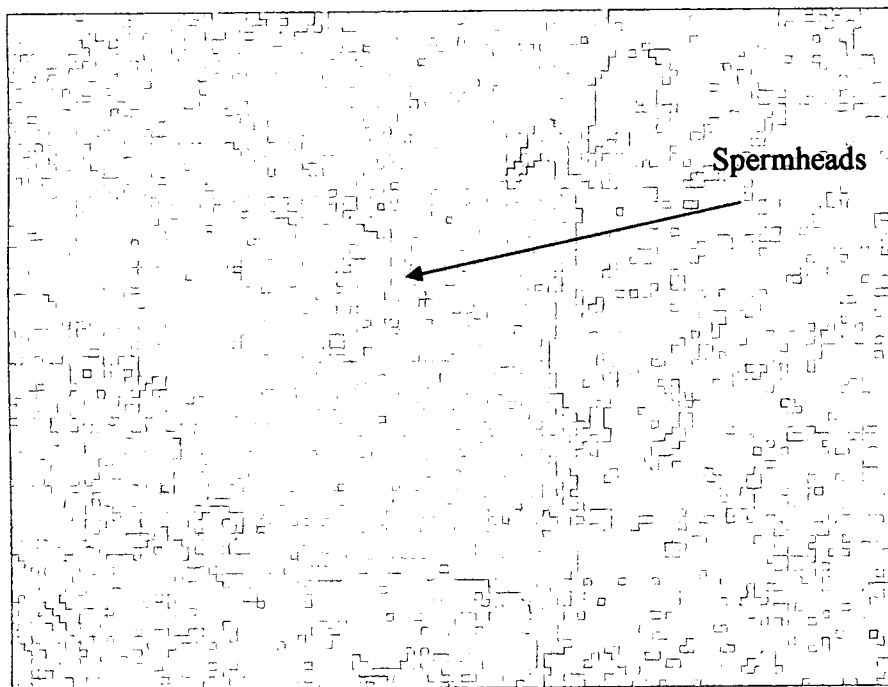


Figure 15: Alignment of the spermheads. 2X magnification of figure 14. The arrow is indicating the spermheads. The 64 sperm appear to be aligned and shaped correctly. This indicates that TGARC supported functional meiotic spindles.

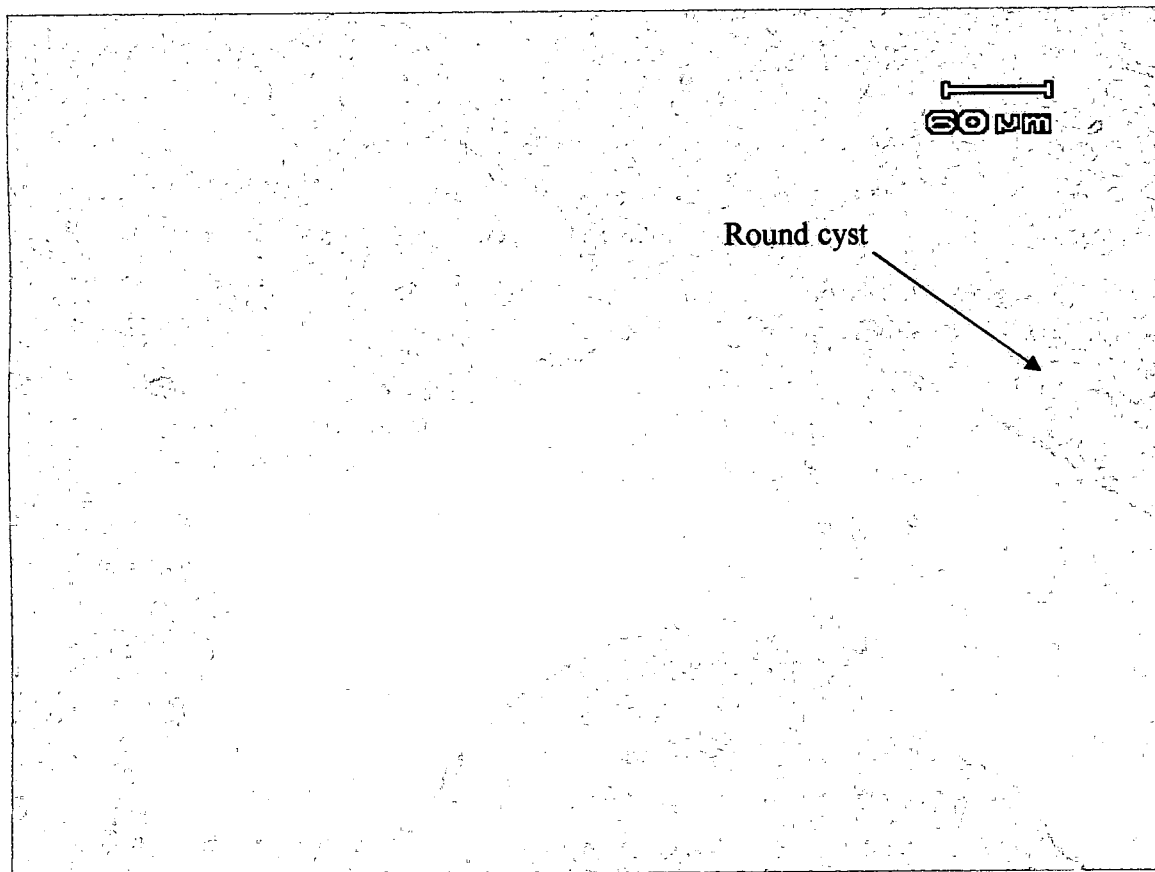


Figure 16: Fly with no sperm. Light micrograph viewed under phase contrast of a male fly with $\beta 2$ null/ $\beta 2$ null. In the absence of $\beta 2$, the spermatids do not elongate and the cysts remain round.

Table 1. Description of chromosomes with dominant phenotypic markers used in genetic crosses.

Genotype	Chromosome location	Phenotype
w ¹¹¹⁸	X	White eyes
Δ 2-3	Third	Source of transposase gene
TM3 (balancer)	Third	Short, stubby bristles
CXD (balancer)	Third	4 bristles, parallel wings
K	Third	β 2 null, ebony body, radius incompletus

Table 2. Genetic crosses used to determine location of P-element insert.

Genotype	Phenotype	Location
F1: P/w; +/+; +/TM3 +/CXD	red eyes, stubby bristles OR parallel wings -NEVER BOTH	Can be on the third
F1: P/w; +/+; TM3/CXD	red eyes, stubby bristles and parallel wings	Cannot be on third
F1: y/w; +/+; TM3/CXD	White eyed males NEVER see red eyed male	Must be on the X
F1: P/y; +/+; TM3/CXD	Red eyed males with both stubby bristles and parallel wings	Must be on the second

Table 3. List of oligonucleotide primers to be used in the site-directed mutagenesis of $\beta 2$ tubulin. Sequence in black is $\beta 1$, while sequence in blue is $\beta 2$. The first three primers will be used to change the nucleotide domain and the last two will change the intermediate domain from $\beta 1$ to $\beta 2$ identity.

Primer 1 18, 23, 29, 37	C CAA ATC GGC GGC AAG TTC TGG GAG GTC ATC TCC GAT GAG CAT TGC ATC GAT GCC ACC GGC GCC TAC TAC GGT GAC AGC
Primer 2 55, 57, 64	C AAT GAG GCG ACC GGT GCC AAG TAC GTG CCC CGC GCT ATC CTT GTC GAT CTG G
Primer 3 124, 126, 135	C CGC AAG GAG TCC GAA GGC TGC GAC TGC CTG CAA GGC TTC CAA AGC ACA CAC TCC
Primer 4 340, 349	C CAG AAC AAG AAC AGC TCC TTC TTC GTC GAA TGG ATC CCC AAC AAC TGC AAG ACC GCC G
Primer 5 381	G CTG TTC AAG CGC GTC TCC GAG CAG TTC ACC

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