

# Mutations in $\alpha$ -tubulin promote basal body maturation and flagellar assembly in the absence of $\delta$ -tubulin

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## Summary

We have isolated suppressors of the deletion allele of  $\delta$ -tubulin, *uni3-1*, in the biflagellate green alga *Chlamydomonas reinhardtii*. The deletion of  $\delta$ -tubulin produces cells that assemble zero, one or two flagella and have basal bodies composed primarily of doublet rather than triplet microtubules. Flagellar number is completely restored in the suppressed strains. Most of the *uni3-1* suppressors map to the *TUA2* locus, which encodes  $\alpha_2$ -tubulin. Twelve independent *tua2* mutations were sequenced. Amino acids D205 or A208, which are nearly invariant residues in  $\alpha$ -tubulin, were altered. The *tua2* mutations on their own have a second phenotype – they make the cells colchicine supersensitive. Colchicine

supersensitivity itself is not needed for suppression and colchicine cannot phenocopy the suppression. The suppressors partially restore the assembly of triplet microtubules. These results suggest that the  $\delta$ -tubulin plays two roles: it is needed for extension or stability of the triplet microtubule and also for early maturation of basal bodies. We suggest that the mutant  $\alpha$ -tubulin promotes the early maturation of the basal body in the absence of  $\delta$ -tubulin, perhaps through interactions with other partners, and this allows assembly of the flagella.

Key words: Basal body, Centriole, Microtubule, Early maturation

## Introduction

Basal bodies are found at the proximal end of cilia and flagella. The core structure of a typical basal body or mature centriole consists of nine triplet microtubules arranged radially in a cartwheel fashion (Manton and Clark, 1952; Fawcett and Porter, 1954) (reviewed in Lange et al., 2000). Basal bodies are required for the construction of eukaryotic cilia or flagella in two ways: as templates for the doublet microtubules of cilia or flagella (Preble et al., 2000) and as docking sites for the molecular motor involved in transport of complexes of flagellar subunits (Cole et al., 1998; Deane et al., 2001).

Basal bodies and centrioles originate in a ring of amorphous material (Dippell, 1968; O'Toole et al., 2003). The triplet microtubules of the blade of a typical centriole or basal body are known as the A, B and C tubules. The C tubule, which is the outermost microtubule, is confined to centrioles and basal bodies (Ringo, 1967), whereas the A and B tubules are continuous with the doublet microtubules of the ciliary or flagellar axoneme. Doublet microtubules are also observed at the distal ends of centrioles (Paintrand et al., 1992) (reviewed in Bornens, 1992).

Mature basal bodies and centrioles usually have triplet microtubules (Gall, 1961; Gould, 1975). However, there are some exceptions. *Caenorhabditis elegans* has centrioles with singlet microtubules at the meiotic spindle poles (Wolf et al., 1968) and *Toxoplasma gondii* has centrioles with singlet microtubules (Morrissette and Sibley, 2002). Immature basal bodies or centrioles may consist of singlet or doublet microtubules (Dippell, 1968; Gaffel, 1988; Chrétien et al.,

1997). *Mastigamoeba schizophrenia*, an anaerobic protozoan, has basal bodies with doublet microtubules that lack the C tubule (Simpson et al., 1997). In *Drosophila melanogaster* embryos, centrioles with doublet microtubules were observed in situ (McDonald and Mophew, 1993; Callaini et al., 1997) isolated centrioles had singlet microtubules (Moritz et al., 1995) and sperm centrioles have triplet microtubules (Mahowald and Strassheim, 1970). The sperm centrioles are longer than the embryonic centrioles in *Drosophila*.

The biflagellate green alga *Chlamydomonas reinhardtii* is an excellent model system for the analysis of basal body and microtubule function (Dutcher, 2000; Dutcher, 2003). The *Chlamydomonas* genome contains two  $\alpha$ -tubulin genes (*TUA1* and *TUA2*), two  $\beta$ -tubulin genes (*TUB1* and *TUB2*), a single  $\gamma$ -tubulin gene (*TUG1*), a single  $\delta$ -tubulin gene (*UNI3*) and a single  $\epsilon$ -tubulin gene (*BLD2*) (Brunke et al., 1984; Silflow et al., 1985; Vassilev et al., 1995; Dutcher and Trabuco, 1998; Dutcher et al., 2002). The two  $\alpha$ -tubulin genes, and the two  $\beta$ -tubulin genes encode identical proteins that are expressed at comparable levels (James et al., 1993; Silflow et al., 1985).

$\delta$ -Tubulin is the fourth member of the tubulin superfamily and was first identified in *Chlamydomonas* (Dutcher and Trabuco, 1998). It is found in the genomes of other organisms with triplet microtubules (Chang and Stearns, 2000; Vaughan et al., 2000; Smrzka et al., 2000) but it is not present in the genomes of *D. melanogaster* (Adams et al., 2000; Celniker et al., 2002) or *C. elegans* (The *C. elegans* Sequencing Consortium, 1998). Cells with the  $\delta$ -tubulin gene deleted (*uni3-1*) have flagellar, basal body and cell division defects.

Electron microscopy of *uni3-1* cells showed a loss of the C tubule along the length of the basal body (Dutcher and Traubauco, 1998). Using gene silencing, a similar lack of the C tubule was observed in *Paramecium tetraurelia* (Garreau de Loubresse et al., 2001).

The *uni3-1* mutant cells have a heterogeneous flagellar phenotype. 50% of *uni3-1* cells are aflagellate, 25% are uniflagellate and 25% are biflagellate. A pedigree analysis of *uni3-1* mitotic cells suggests that the ability to assemble a flagellum from a basal body lacking  $\delta$ -tubulin requires maturation of the basal body through multiple cell cycles (Dutcher and Traubauco, 1998). These observations raise several important questions about the roles of  $\delta$ -tubulin and the C tubule. First, if the C tubule is needed to assemble flagella, why are *uni3-1* cells able to assemble flagella in a sizeable fraction of the cells? Second, if  $\delta$ -tubulin is needed to assemble the C tubule, why do sperm basal bodies in *Drosophila*, which lack  $\delta$ -tubulin, have C tubules?

To address these questions, we screened for suppressors of the *uni3-1* deletion allele in *Chlamydomonas*. We report here the isolation and characterization of 12 *tua2* alleles (encoding  $\alpha_2$ -tubulin) that suppress the flagellar assembly defects of the *uni3-1* allele. Only two different mis-sense mutations in *TUA2* were found among these 12 independent alleles. We observed that these mis-sense mutations in  $\alpha$ -tubulin can partially restore C-tubule assembly. This result suggests that  $\delta$ -tubulin is not strictly required for C-tubule assembly. We suggest that the mutant  $\alpha$ -tubulin alters the ability of the basal body to undergo maturation, perhaps through interactions with other partners.

## Materials and Methods

### Cell culture and genetic analysis

Culture conditions and media were as described previously (Lux and Dutcher, 1991) and nitrite medium was as described by Fernández et al. (Fernández et al., 1989). Standard matings were as described in Dutcher (Dutcher, 1995). Diploid strains were selected as described (Palombella and Dutcher, 1998) using the linked *nit2-1* and *ac17* mutations in trans for selection. The *pyr1* and *tub2-1* (formerly *col<sup>R4</sup>*) mutant strains were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC) and the *tua1-1* and *apm1-1* mutations were obtained from P. Lefebvre (University of Minnesota, Minneapolis, MN). All drugs were added after autoclaving. Stock solutions of colchicine were made in ethanol and stored in the dark. Medium with colchicine was incubated in yellow lucite boxes or yellow Saran Wrap to prevent breakdown of the colchicine (Palombella and Dutcher, 1998). Concentrations of colchicine ranged from 0.10 mM to 4.5 mM. Pyrythiamine was used at 1  $\mu$ M. 5-fluorindole was used at 7  $\mu$ M and assayed in yellow lucite boxes to prevent its breakdown (Palombella and Dutcher, 1998). Cells were assayed on medium with oryzalin and vinblastine from 1  $\mu$ M to 15  $\mu$ M. Oryzalin was a gift from Eli Lilly Laboratories (Indianapolis, IN). Stock solutions of these drugs were solubilized in 100% dimethyl sulfoxide (DMSO). Measurements of cell sizes were performed as described (Preble et al., 2001).

### Isolation of suppressors of *uni3-1*

Two separate screens were used to isolate cells that regained the ability to assemble two flagella on most cells. In the first screen, 180 single *uni3-1* colonies were grown for 3 days on R medium and then subjected to ultraviolet (UV) irradiation for 45 seconds (Lux and Dutcher, 1991). The irradiated colonies were allowed to recover in the dark for 24 hours and were then placed individually

into 20 ml R medium in 150 mm culture tubes. Cells that swam to the upper 5 ml were transferred. This transfer was repeated seven times. 10  $\mu$ l of supernatant was plated to solid R medium, 15–20 individual colonies were picked into 2.5 ml R medium and assayed for swimming ability, and 48 suppressed strains were recovered. In the second screen, 200 single *uni3-1* colonies were grown for 3 days on R medium and then subjected to  $\gamma$ -irradiation for 30 minutes from a  $^{137}\text{Cs}$  source (498 Roentgen/minute). The same enrichment protocol described above was followed and 15 colonies were recovered.

The first 18 strains were mated to a wild-type parent (CC124) and the segregation of swimming phenotypes was assayed. All strains contained extragenic suppressors that were easily separated from the *uni3-1* mutation by recombination. The remainder of the strains were mated to a *pyr1* strain because we had determined that most suppressors were linked to this mutation. This provided a quick assay for placing strains into recombination groups. Further classification of the strains was based on complementation and recombination tests using suppression and colchicine supersensitivity as assays. Diploid strains that were homozygous for the *uni3-1* allele were used for suppression assays and diploid strains that were homozygous for the *UNI3* allele were used for colchicine-supersensitivity assays.

### Isolation of revertants of *tua2* alleles

Because the *tua2* alleles confer supersensitivity to colchicine, it was possible to select revertants on media with low concentrations of colchicine. Revertants of the supersensitivity phenotype of *tua2-1*, *tua2-2*, *tua2-3* and *tua2-4* strains were selected. Approximately  $10^8$  cells were placed on 1.75 mM and 2.2 mM colchicine plates without mutagenesis and grown for 2–3 weeks. A single revertant was saved from each plate and retested on media with a range of colchicine concentrations. Each strain was mated to a *pyr1* parent and the segregation of the reversion was determined. All events in the five strains were inseparable from the supersensitive phenotype. The revertants were named *tua2-1R1*, *tua2-2R1*, *tua2-3R1* and *tua2-4R1*. Each revertant was assayed for growth phenotypes and flagellar assembly. Each revertant strain was mated to *uni3-1* cells to determine whether suppression of the flagellar assembly phenotype of *uni3-1* was also reverted. From each of the crosses *tua2-1R1* $\times$ *TUA2*, *tua2-2R1* $\times$ *TUA2*, *tua2-3R1* $\times$ *TUA2* and *tua2-4R1* $\times$ *TUA2*, 12 meiotic progeny were subsequently mated to *uni3-1 pyr1 mt<sup>+</sup>* or *uni3-1 pyr1 mt<sup>-</sup>* strains to verify the failure to suppress the flagellar phenotype of *uni3-1* cells and to verify new synthetic phenotypes.

### Drug sensitivity tests

To determine the degree of colchicine supersensitivity, cells were grown to densities of  $\sim 2 \times 10^6$  cells ml<sup>-1</sup>. The cells were diluted 1000-fold and then plated on solid R medium to determine the number of cells that could form colonies. The data are displayed graphically as the percentage of viable cells against the concentration of colchicine.

### Polymerase chain reaction and DNA sequencing

Genomic DNA was isolated as previously described (Dutcher and Traubauco, 1998). The  $\alpha_2$ -tubulin gene was amplified using the *TUA2*-specific primers A2-1 (CCAACAACACTCCACAAA) and A2-2 (CGAAGCCTCAGATCACGCCGCACAGGA), generating an  $\sim 2$  kb product. This PCR product contained the entire coding region, introns and 5' and 3' untranslated sequences of *TUA2*. PCR reactions were carried out in 100  $\mu$ l volumes, containing 40 pmol of each primer, 20 nmol mixed dNTPs, 50–100 ng genomic DNA, 10 mM Tris (pH 8.8), 1.5 mM MgCl<sub>2</sub>, 25 mM KCl and 2.5 U Taq DNA polymerase (Gibco BRL; Boehringer-Mannheim). PCR cycling conditions were: 95°C 5 minutes once, followed by 35 cycles of 95°C for 1 minute, 55°C for 2 minutes, 72°C for 2 minutes, and a final annealing cycle of 95°C for 1 minute, 55°C for 2 minutes and 72°C for 5 minutes.

The PCR products were isolated from 1% agarose gels using the GeneClean method (Bio101, La Jolla, CA) and sequenced using A2-1, A2-2 and internal primers using the DNA Sequencing Facility of Iowa State University.

**Protein preparations**

Cell bodies were separated from axonemes by centrifugation after deflagellation with dibucaine (King, 1995) and were passed twice through a French Press at 4000 psi. Insoluble material was removed by centrifugation at 34,000 g for 60 minutes. Axonemal proteins were prepared as described (King, 1995), with the addition of Nonidet P-40 to 0.1%. Protein concentrations were determined using the BCA reagent (Sigma, St. Louis, MO) with bovine serum albumin (BSA) as a standard. Denaturing electrophoresis was performed on 10% polyacrylamide vertical minigels Mini V 8•10 (Gibco BRL/Life Technologies) according to Laemmli (Laemmli, 1970). Gels were transferred to nitrocellulose by using the Gibco BRL/Life Technologies Mini-V 8•10 Vertical Gel Electrophoresis system for 1 hour at 100 V as recommended by the manufacturer. After transfer, the nitrocellulose blots were first blocked with 3% BSA in TBS (Bollag et al., 1996) and subsequently probed with a rabbit polyclonal antibody to α-tubulin (kindly provided by D. Cole) (Cole et al., 1998) at 1:10,000 in 1.5% BSA in TBS, with a monoclonal antibody to α-tubulin (DMA1) (Research Diagnostics, Flanders, NJ) at 1:1000 in 10% goat serum in PBS, and a monoclonal antibody to acetylated α-tubulin (LeDizet and Piperno, 1986). A secondary goat anti-rabbit polyclonal antibody or a secondary goat anti-mouse polyclonal antibody (Biorad, Hercules, CA) conjugated to alkaline phosphatase was used at a 1:2000 dilution to detect primary antibody binding.

**Threading *Chlamydomonas* sequence onto the crystal structure of tubulin**

The amino acid sequences of *Chlamydomonas* α- and β-tubulin (accession number A523928 and UKBM, respectively) were matched to the crystal structure of bovine tubulin (accession number 1JFF) using the Swiss-Model Automated Comparative Protein Modeling Server (<http://www.expasy.ch/swissmod/>) in the first approach mode. The resulting structure was viewed by SwissPdb Viewer version 3.7 (<http://www.expasy.ch/spdbv/>).

**Electron microscopy**

Cell pellets were prepared for electron microscopy by high-pressure freezing followed by freeze substitution as described (Preble et al., 2001; O'Toole et al., 2003). Serial thin sections, of 50-60 nm were viewed in a Philips CM10 electron microscope operating at 80 kV or 100 kV. Using the microscope's goniometer stage, the sections were tilted to an angle that permitted the basal body microtubules to be viewed in cross section. To allow tilting along the appropriate axis, a rotating specimen holder was used.

**Results**

**Suppressors of *uni3-1* are linked to *TUA2***

We generated suppressors of the *uni3-1* deletion allele by treating *uni3-1* cells with UV or γ-irradiation. Because 25% of the cells in *uni3-1* cultures are biflagellate and can swim effectively, suppressors could be identified as cultures with an increased proportion of swimming cells. Sixty-one suppressed strains were isolated from 254 independent cultures. Forty-six of the suppressor alleles are linked to the *TUA2* locus (α<sub>2</sub>-tubulin) on linkage group IV based on mapping to *pyr1*, which confers resistance to pyriithiamine (Ranum et al., 1988; Harris, 1989) (Table 1). Eleven of the

**Table 1. Genetic characterization of *uni3-1* suppressors**

Isolation name	Allele name	Mutagen	Segregation with <i>pyr1</i> (PD:NPD:TT)	Segregation with <i>tua2-3</i> (PD:NPD:TT)	Molecular defect
UV7	<i>tua2-1</i>	UV	53:0:11	98:0:0	D <sub>205</sub> N
UV29	<i>tua2-2</i>	UV	35:0:7	130:0:0	D <sub>205</sub> N
UV56	<i>tua2-3</i>	UV	36:0:7	56:0:0	D <sub>205</sub> N
UV82	<i>tua2-4</i>	UV	58:0:10	154:0:0	D <sub>205</sub> N
UV84	<i>tua2-5</i>	UV	25:0:7	125:0:0	D <sub>205</sub> N
UV100	<i>tua2-6</i>	UV	23:0:6	87:0:0	D <sub>205</sub> N
UV120	<i>tua2-7</i>	UV	24:0:7	89:0:0	A <sub>208</sub> T
γ-24	<i>tua2-8</i>	γ	26:0:8	95:0:0	A <sub>208</sub> T
γ-45	<i>tua2-9</i>	γ	59:0:12	34:0:0	A <sub>208</sub> T
γ-54	<i>tua2-10</i>	γ	24:0:6	56:0:0	D <sub>205</sub> N
γ-60	<i>tua2-11</i>	γ	23:0:10	123:0:0	D <sub>205</sub> N
γ-66	<i>tua2-12</i>	γ	25:0:5	146:0:0	D <sub>205</sub> N
γ-78	<i>tua2-13</i>	γ	24:0:8	34:0:0	NT
γ-90	<i>tua2-14</i>	γ	15:0:4	18:0:0	NT
γ-110	<i>tua2-15</i>	γ	14:0:5	22:0:0	NT
γ-133	<i>tua2-16</i>	γ	15:0:6	19:0:0	NT
γ-145	<i>tua2-17</i>	γ	14:0:3	7:0:0	NT
γ-147	<i>tua2-18</i>	γ	13:0:4	21:0:0	NT
γ-168	<i>tua2-19</i>	γ	14:0:4	24:0:0	NT
γ-178	<i>tua2-20</i>	γ	12:0:4	17:0:0	NT
γ-182	<i>tua2-21</i>	γ	13:0:5	14:0:0	NT
γ-195	<i>tua2-22</i>	γ	14:0:2	15:0:0	NT
UV4a	<i>tua2-23</i>	UV	15:0:3	29:0:0	NT
UV6a	<i>tua2-24</i>	UV	18:0:4	12:0:0	NT
UV14	<i>tua2-25</i>	UV	17:0:3	21:0:0	NT
UV22	<i>tua2-26</i>	UV	13:0:5	NT	NT
UV50	<i>tua2-27</i>	UV	12:0:4	NT	NT
UV55	<i>tua2-28</i>	UV	18:0:4	NT	NT
UV56	<i>tua2-29</i>	UV	19:0:5	NT	NT
UV62	<i>tua2-30</i>	UV	14:0:5	NT	NT
UV77	<i>tua2-31</i>	UV	12:0:5	NT	NT
UV79	<i>tua2-32</i>	UV	18:0:5	NT	NT
UV86	<i>tua2-33</i>	UV	17:0:3	NT	NT
UV97	<i>tua2-34</i>	UV	17:0:2	NT	NT
UV98	<i>tua2-35</i>	UV	18:0:4	NT	NT
UV99	<i>tua2-36</i>	UV	17:0:4	NT	NT
UV102	<i>tua2-37</i>	UV	17:0:4	NT	NT
UV105	<i>tua2-38</i>	UV	18:0:1	NT	NT
UV108	<i>tua2-39</i>	UV	19:0:4	NT	NT
UV110	<i>tua2-40</i>	UV	18:0:3	NT	NT
UV111	<i>tua2-41</i>	UV	19:0:4	NT	NT
UV112	<i>tua2-42</i>	UV	18:0:3	NT	NT
UV115	<i>tua2-43</i>	UV	21:0:4	NT	NT
UV116	<i>tua2-44</i>	UV	17:0:4	NT	NT
UV117	<i>tua2-45</i>	UV	19:0:3	NT	NT
UV125	<i>tua2-46</i>	UV	24:0:1	NT	NT

PD: NPD: TT, number of parental ditype to nonparental ditype to tetraplye tetrads observed; NT, not tested.

remaining 16 suppressors map to linkage group II and show no phenotypes beyond suppression of *uni3-1*. The remaining four suppressors do not map to known tubulin loci and also convey supersensitivity to colchicine. Further analysis of the remaining recombination groups will be reported elsewhere. Each of the 46 strains showed complete suppression of the flagellar assembly defect: more than 92% of the suppressed cells had two flagella, 3% of the cells had more than two flagella and the remainder were aflagellate (*n*=200). Eight of the 46 alleles were tested for dominance and were found to be recessive for suppression of the flagellar defects of the *uni3-1* allele (Table 2). These alleles did not suppress the uniflagellate phenotypes of the *uni1* or *uni2* alleles (data not shown) (Huang et al., 1982; Dutcher, 1986; Dutcher and

**Table 2. Complementation tests for suppression of the uniflagellar phenotype of *uni3-1***

Genotype of parent 1 ( <i>nit2 AC17</i> )	Genotype of parent 2 ( <i>NIT2 ac17</i> )							
	<i>tua2-1</i>	<i>tua2-2</i>	<i>tua2-3</i>	<i>tua2-4</i>	<i>tua2-5</i>	<i>tua2-6</i>	<i>tua2-7</i>	<i>tua2-9</i>
<i>TUA2</i>	+	+	+	+	+	+	+	+
<i>tua2-1</i>	-	-	-	-	NT	-	-	-
<i>tua2-2</i>	-	-	-	-	-	-	-	-
<i>tua2-3</i>	-	-	-	-	NT	-	-	-
<i>tua2-4</i>	-	-	-	-	-	-	-	-
<i>tua2-5</i>	-	-	-	-	NT	-	-	-
<i>tua2-6</i>	-	-	-	-	-	-	-	-
<i>tua2-7</i>	-	-	-	-	-	-	-	-
<i>tua2-9</i>	-	-	-	-	-	-	-	-

+, suppression is recessive to the wild-type *TUA2* allele. Cells have zero, one or two flagella; -, indicates the failure to complement the suppression phenotype. Diploid strains are homozygous for the *uni3-1* allele and >95% of the cells have two flagella. NT, not tested.

Trabuco, 1998). When the 46 suppressors were examined in *UNI3* strains, they formed functional, motile flagella. In addition, they showed supersensitivity to the tubulin-binding drug, colchicine (Fig. 1). Colchicine supersensitivity is recessive to the wild-type allele in 15 strains examined at colchicine concentrations ranging from 0.1 mM to 1.0 mM (Table 3). These mutations do not confer supersensitivity to the other microtubule-affecting drugs oryzalin, isophenyl carbamate or vinblastine or confer resistance to Taxol (data not shown). Supersensitivity to antimicrotubule-agents can be conferred by mutations in  $\alpha$ - or  $\beta$ -tubulin as well as other genes (Umesono et al., 1983; Hoyt et al., 1990; Jung et al., 1990; Stearns et al., 1990; Chen et al., 1994). The genetic map position and colchicine-supersensitivity phenotype conferred by these mutations suggested that these suppressors carried mutations in *TUA2*.

#### Suppressors of *uni3-1* are missense mutations in *TUA2*

In order to determine whether these suppressors are mutated in *TUA2*, we isolated genomic DNA from 12 of the 46 suppressor strains and amplified the *TUA2* gene by PCR. The isolated PCR products were then directly sequenced. *TUA2* DNA from three control strains (137c *mt*<sup>+</sup>, *uni3-1* and one unlinked suppressor) was amplified and sequenced.

The *TUA2* sequences from mutant and control strains contain 12 base changes in intron 1 (GenBank Accession number AY182002) compared with the sequence deposited in GenBank (Accession number M11448) (Silflow et al., 1985) and modified in James (James et al., 1993). These changes include nucleotide substitutions and insertions. The parental strain used in our laboratory (137c) is derived from a different isolate of *Chlamydomonas* than that used in the previous studies of *TUA2* (Harris, 1989), and so we attribute the intron 1 changes to genetic drift. No additional base changes were found in the three control sequences. However, when we compared the sequences of the *pyr1*-linked suppressor strains with the parental strain, we found one of two nucleotide substitutions in exon 3.

Nine strains were found to have an A instead of a G at position 1210, which results in the amino acid change D205N. Three strains have an A instead of a G at position 1219, which results

**Table 3. Complementation tests for colchicine-supersensitivity phenotype**

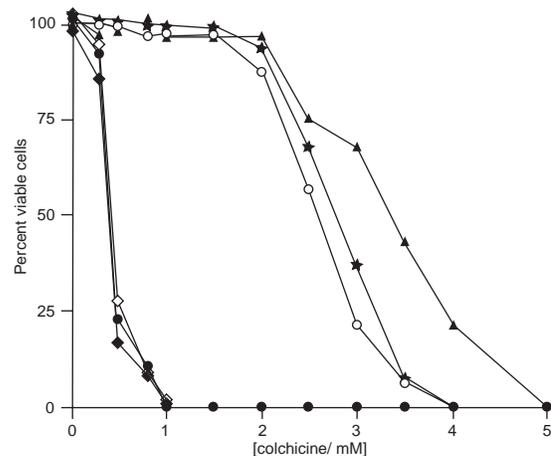
Genotype of parent 1 ( <i>nit2 AC17</i> )	Genotype of parent 2 ( <i>NIT2 ac17</i> )				
	<i>TUA2</i>	<i>tua2-2</i>	<i>tua2-3</i>	<i>tua2-7</i>	<i>tua2-9</i>
<i>tua2-1</i>	+	-	-	-	-
<i>tua2-2</i>	+	-	-	NT	-
<i>tua2-3</i>	+	NT	-	-	-
<i>tua2-4</i>	+	NT	-	-	-
<i>tua2-5</i>	+	NT	-	-	-
<i>tua2-6</i>	+	NT	-	-	-
<i>tua2-7</i>	+	NT	-	-	-
<i>tua2-8</i>	+	NT	-	-	-
<i>tua2-9</i>	+	NT	-	-	-
<i>tua2-10</i>	+	NT	-	NT	-
<i>tua2-11</i>	+	NT	-	NT	-
<i>tua2-12</i>	+	NT	-	NT	NT
<i>tua2-13</i>	+	NT	-	NT	NT
<i>tua2-14</i>	+	NT	-	NT	-
<i>tua2-15</i>	+	NT	-	NT	-

+, diploid cells that grow on 1.0 mM colchicine medium and with recessive supersensitivity phenotype; -, diploid cells that fail to grow on 1.0 mM colchicine medium and with allelic mutations; NT, not tested.

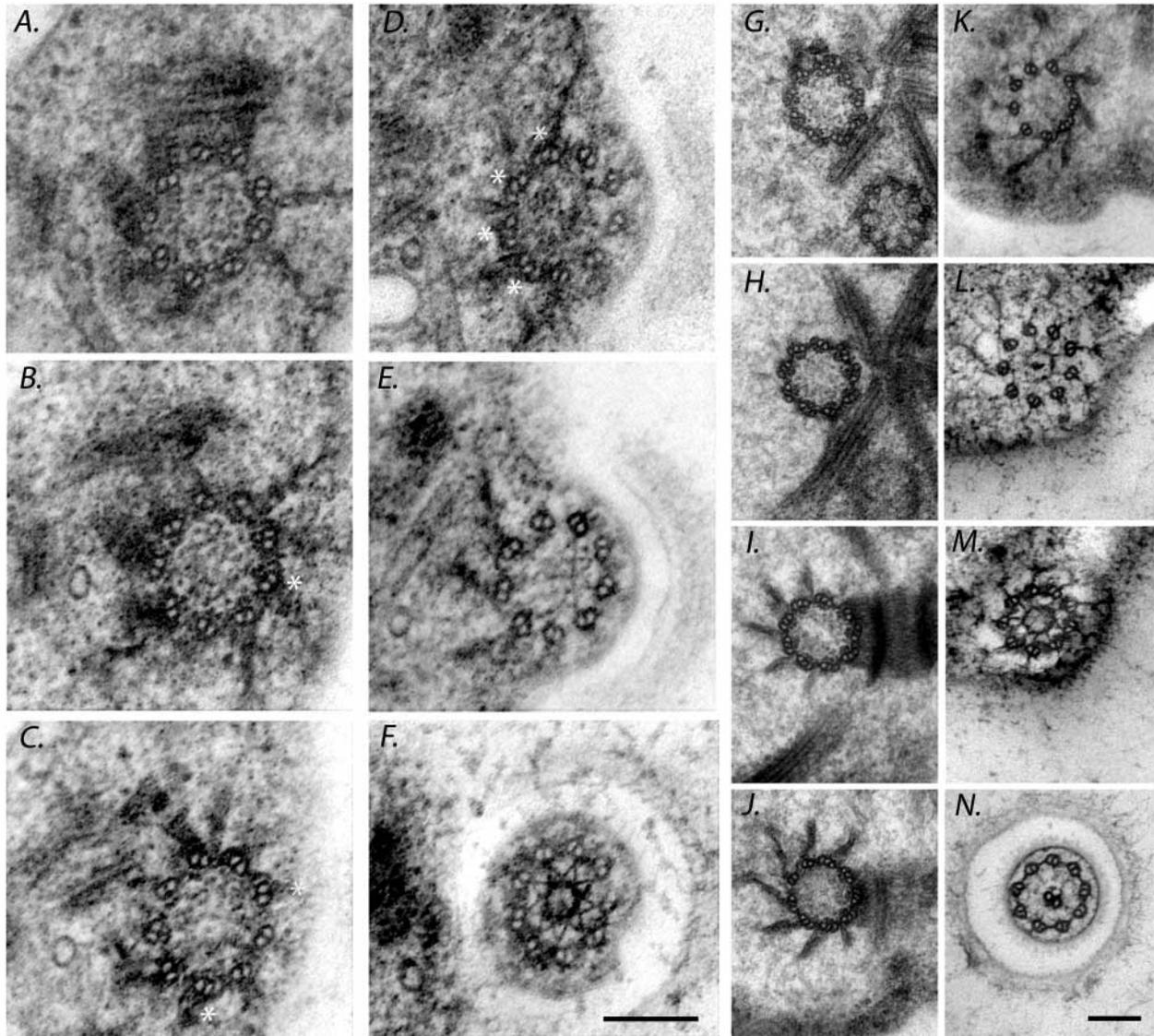
in the amino acid change A208T. We verified that these changes were not due to errors made by Taq DNA polymerase by sequencing the PCR products from multiple independent reactions. In addition, we sequenced 125 bps of *TUA2* DNA (including nucleotide 1210) from 12 meiotic progeny from a cross of *tua2-1* × *TUA2* and found that the colchicine phenotype cosegregates with the base change at position 1210 in all progeny tested. Because the suppressors are clearly *TUA2* alleles, they have been named *tua2-1* through to *tua2-12* (Table 1).

#### Basal bodies of *tua2*; *uni3-1* cells assemble some triplet microtubules

We found previously that *uni3-1* cells have doublet microtubules in their basal bodies. However, small stretches of



**Fig. 1.** Survival of cells on varying concentrations of colchicine. The percentage of viable cells is plotted against the different concentrations of colchicine. ♦, *tua2-1*; ●, *tua2-7*; ◇, *tua2-1*; *uni3-1*; ○, wildtype; ★, *TUA2*; *uni3-1*, ▲, *tub2-1*. Strains with a *tua2* mutation are more sensitive to colchicine than *TUA2* strains. Cells with *tub2-1* mutation in  $\beta_2$ -tubulin are more resistant to colchicine.



**Fig. 2.** Serial section electron microscopic images, showing cross-sections of the basal body region of *tua2-6; uni3-1* (A-F) and two different *tua2-6; UNI3* cells (G-J and K-N). Scale bars 0.4  $\mu\text{m}$ . (A) Distal region of a basal body with doublet microtubules. (B,C) Distal region of a basal body with doublet and triplet microtubule blades, triplet blades are indicated by asterisks. This basal body has three extended triplet blades. (D) Distal region of a basal body with both doublet and triplet microtubule blades, triplet blades are indicated by asterisks. Most of the triplet blades are only present at the distal end of the basal body. (E,F) Transition zone of a basal body as indicated by the stellate fiber. As in wild-type basal bodies, only doublet microtubule blades are observed in the transition zone. (G) A mature basal body is present and a probasal body (lower right) with triplet microtubule blades; the angles of the blades are indicative of a probasal body rather than of a mature basal body. (H) A mature basal body and probasal body are separated by rootlet microtubules that form a cross-shaped pattern. (I) A mature basal body with the distal striated fibers that are present only at the distal end. Transition fibers emanate from the basal body at the distal end. (J) Transition fibers elongate in a more distal region. The majority of the blades still have triplet microtubules. (K) Triplet microtubules have become doublet microtubules. Transition fibers are still present and are elongated on the right of the basal body, but on the left they have become connectors to the membrane. (L) Doublet microtubules with connectors to the membrane. (M) Transition zone with the appearance of the central stellate fibers that form a central ring. (N) Outer doublet microtubules and central pair microtubules of the flagellar axoneme.

the C tubule have been found at the distal end of the basal bodies from *uni3-1* cells using dual-axis electron tomography of basal bodies (O'Toole et al., 2003). We observed a modest increase in the frequency and length of C tubules in *uni3-1; tua2-6* basal bodies compared with *uni3-1* basal bodies. The tendency of *uni3-1* basal bodies to form a second stellate fiber array (normally located in the transition zone between the basal body

and the axoneme, in the proximal half of the basal body) was retained in *tua2-6; uni3-1* basal bodies (O'Toole et al., 2003).

In parallel with the above study, we examined serial sections of *tua2-6; uni3-1* cells to quantify the rescue by the *tua2-6* suppressor. We found that basal bodies had acquired up to nine triplet microtubules (one example in Fig. 2A-F). One of the 14 serially sectioned basal bodies had nine triplet microtubules

**Table 4. Genetic analysis of intragenic revertants of *tua2* alleles**

Intragenic revertant	Segregation in wild-type crosses (PD: NPD: TT)	Suppression of <i>uni3-1</i> cells	Molecular lesion
<i>tua2-1R1</i>	167:0:0	No suppression	None found in coding region
<i>tua2-2R1</i>	178:0:0	No suppression	7 bp deletion/frameshift
<i>tua2-3R1</i>	210:0:0	Enhancement, no flagella	P173L
<i>tua2-4R1</i>	150:0:0	No suppression	None found in coding region

whereas the remainder had one ( $n=2$ ), two ( $n=3$ ), three ( $n=5$ ) or four ( $n=4$ ) triplets. Thus, suppression of the flagellar assembly defect does not appear to arise from the assembly of basal bodies with a full complement of nine triplet microtubules. Suppression might result from the limited restoration of triplet microtubules within a basal body or from the altered properties of the mutant  $\alpha$ -tubulin. As observed by tomography (O'Toole et al., 2003), stellate fibers were present in the interior of proximal regions of the basal bodies. We also examined serial sections of 13 *tua2-6*; *UNI3* basal bodies and found no differences from wild-type cells. Serial sections of two *tua2-6*; *UNI3* cells are shown in Fig. 2G-N. Structures along the basal body are indistinguishable from wild-type basal bodies.

#### The cleavage furrow defect of *uni3-1* is not suppressed by the *tua2* alleles

In wild-type populations, two recently divided daughter cells have nearly equal sizes (Preble et al., 2001). Multiple mutant alleles in the gene for  $\epsilon$ -tubulin have defects in the placement of the spindle and cleavage furrow (Ehler et al., 1995; Preble et al., 2001; Dutcher et al., 2002). Previous observations of *uni3-1* cells suggested that their cleavage furrow is not placed normally (Dutcher and Trabuco, 1998). To confirm this observation, the areas of pairs of daughter cells that had recently divided were measured for wild-type, *uni3-1*, *tua2-2*, *tua2-6*, *tua2-2*; *uni3-1* and *tua2-6*; *uni3-1* cells, and the ratio between the large and the small cell was calculated. For wild-type cells this ratio is close to 1. The distribution of the ratio of large to small *uni3-1* daughter cells is broader than for wild-type cells (Fig. 3A,B). The distribution of the ratio of large to small daughter cells for *tua2-2*; *uni3-1* and *tua2-6*; *uni3-1* is similar to that for *uni3-1* cells (Fig. 3B-D). The distributions of the ratios of *tua2-2* and *tua2-6* cells (Fig. 3E,F) resemble wild-type cells. Although the *tua2-2* and *tua2-6* alleles suppress the flagellar defects of the *uni3-1* allele completely, they do not suppress the defects in cleavage furrow placement.

#### Isolation of intragenic revertants of *tua2*

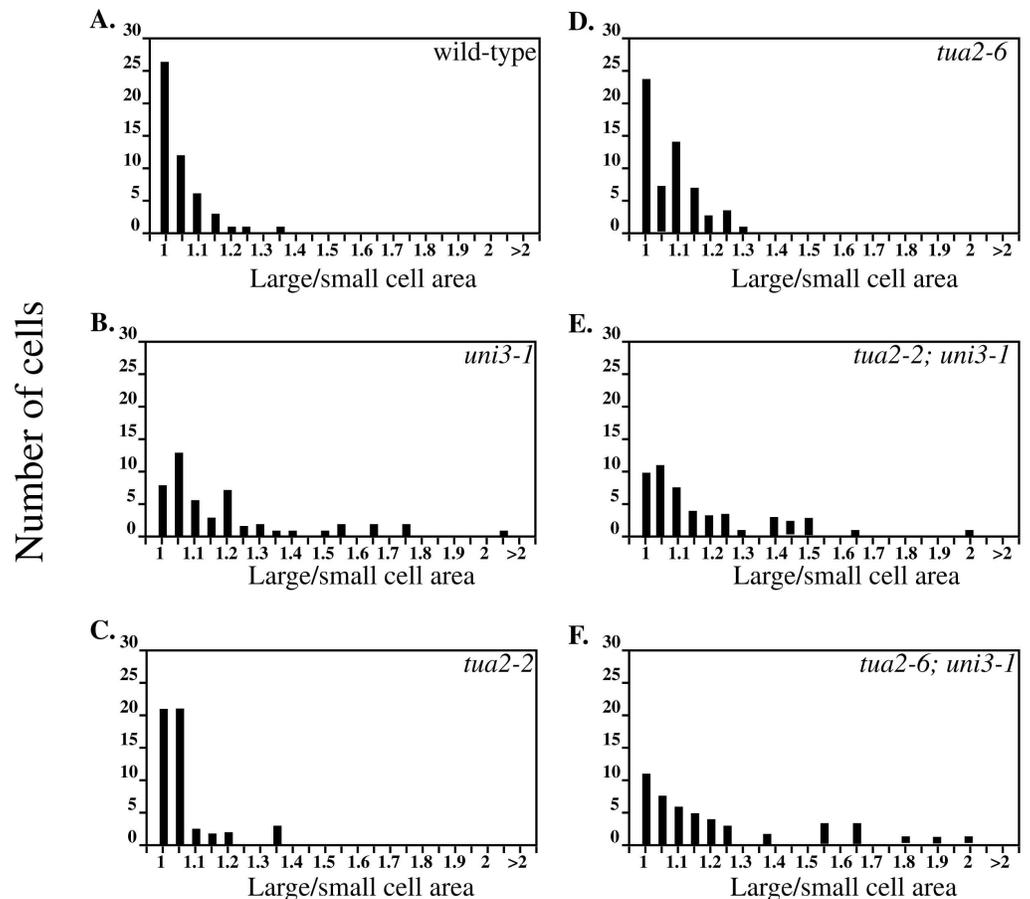
Does reversion of the colchicine-supersensitivity phenotype of the *tua2* alleles also result in reversion of the ability to suppress the flagellar phenotype of *uni3-1*? To answer this question, we isolated four spontaneous revertants of several *tua2* alleles with the D205N amino acid change that enables them to form colonies on 1.0  $\mu$ M colchicine. Each of the revertants was

**Table 5. Dominance tests of intragenic revertants with *tua2* and *TUA2* alleles**

Genotype of diploid cells	Colchicine phenotype (1 mM)	Flagellar phenotype
<i>tua2-1R1/tua2-1</i>	Sensitive	Wild type
<i>tua2-2R1/tua2-1</i>	Sensitive	Wild type
<i>tua2-3R1/tua2-1</i>	Sensitive	Wild type
<i>tua2-4R1/tua2-1</i>	Sensitive	Wild type
<i>tua2-1R1/TUA2</i>	+	Wild type
<i>tua2-2R1/TUA2</i>	+	Wild type
<i>tua2-3R1/TUA2</i>	+	Wild type
<i>tua2-4R1/TUA2</i>	+	Wild type
<i>tua2-2R1; TUA1/TUA2; tua1-1</i>	+	Wild type
<i>tua2-3R1/tua2-2R1</i>	+	Wild type
<i>tua2-3R1; uni3-1/TUA2; uni3-1</i>	+	Intermediate between <i>uni3-1</i> and <i>UNI3</i>
<i>tua2-3R1; TUA1/TUA2; tua1-1</i>	+	Wild type
<i>tua2-2R1; uni3-1/tua2-2; uni3-1</i>	Sensitive	Wild type

Sensitive, cells fail to grow on 1 mM colchicine medium; +, cells grow on 1 mM colchicine medium.

inseparable from the colchicine-supersensitive phenotype in more than 150 tetrads for each of the four revertant strains (Table 4). These results suggested that the reversion event was likely to be intragenic. No obvious flagellar or cell division phenotypes were observed in the haploid revertant strains. The population doubling times for *tua2-6* and *tua2-12* are similar to that for wild-type cells at 25°C (7.5 hours, 7.6 hours and 7.5 hours, respectively), and the population doubling times for *tua2-6*; *uni3-1*, *tua2-12*; *uni3-1* and *uni3-1* are similar (8.1 hours, 8.0 hours, and 8.2 hours respectively). Diploid cells that are heterozygous for intragenic revertants with a *tua2-1* or a *TUA2* allele were constructed and analyzed. The diploid strains heterozygous for *TUA2* have wild-type levels of sensitivity to colchicine and no flagellar phenotypes (Table 5), whereas the diploid strains heterozygous for *tua2-1* remain supersensitive to colchicine. Thus, the revertant alleles are recessive to the colchicine-supersensitive allele suggesting that they have a loss or a reduction in function. In addition, they do not show any interaction with the *tua1-1* allele in  $\alpha_1$ -tubulin in heterozygous diploid strains. The phenotypes of the revertants in combination with the *uni3-1* allele were examined. The *tua2-1R1*, *tua2-2R1* and *tua2-4R1* alleles lost the ability to suppress the flagellar phenotype of *uni3-1* cells. The *tua2-2R1* allele was recessive to the *tua2-2* allele for suppression of the flagellar defect of *uni3-1*, which again suggests that the revertant allele has a loss-of-function phenotype. The *tua2-3R1* allele had no phenotype of its own, but did show a phenotype in combination with the *uni3-1* allele. The *uni3-1*; *tua2-3R1* double mutant was aflagellate at all temperatures examined when grown vegetatively but was flagellated when grown as gametic cells. Ninety-five percent of the cells had two full-length flagella, and wild-type motility. Thus, *tua2-3R1* acts as an enhancer of the *UNI3* flagellar phenotype. This interaction with the *uni3-1* allele is partially dominant. In *tua2-3R1*; *uni3-1/TUA2*; *uni3-1* cells, 75% of the cells are aflagellate and the remainder are unflagellate, which suggests that a mutant protein is present and interferes with the wild-type  $\alpha$ -tubulin. The diploid phenotype suggests that the presence of three wild-type copies of  $\alpha$ -tubulin allele can compete with the mutant protein.



**Fig. 3.** Compared are ratios of sizes of daughter cells following mitotic cell division in wild-type and mutant cell populations. Histograms display the number of cells with a given large-/small-cell area ratio. *uni3-1* cells produce daughters of unequal sizes, suggesting a defect in the placement of the cleavage furrow. This defect is not suppressed by either of the *tua2* alleles. *tua2* strains show wild-type ratios, which indicates that the positioning of the cleavage furrow is not affected by the mutations.

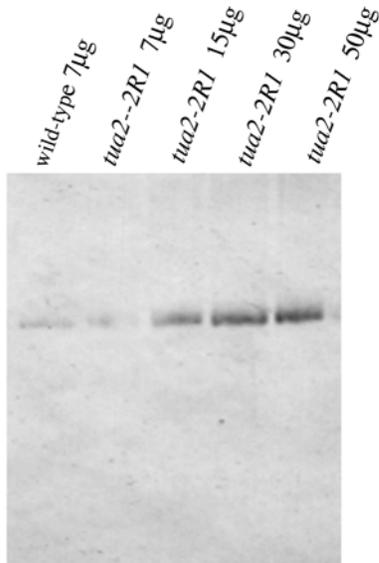
The *TUA2* genes in the four revertants were sequenced. In the *tua2-3R1* allele, we found a single base change in addition to the original lesion. The base change C1115T is predicted to cause the amino acid substitution P173L. We found a complicated molecular lesion in the *tua2-2R1* allele in addition to the original *tua2-2* mutation. A nine-base insertion accompanied by a two-base deletion appears to have occurred and is probably the result of duplicated adjacent DNA. The *tua2-2R1* allele probably encodes an altered and truncated  $\alpha_2$ -tubulin; amino acids 334-339 in wild-type  $\alpha_2$ -tubulin would be missing and the last 106 amino acids would be replaced by 29 'random' amino acids in addition to the original lesion D205N.

The protein encoded by the *tua2-2R1* allele would probably not be functional in microtubule-based structures, even if it was stable in the cell. The predicted molecular mass of this mutant protein is ~36 kDa, which is significantly smaller than the 49 kDa predicted for the wild-type polypeptide. Flagellar proteins from *tua2-2R1* cells and wild-type cells were isolated and analyzed by SDS-PAGE followed by immunoblotting and probed with three anti- $\alpha$ -tubulin antibodies (Cole et al., 1998; Blose et al., 1984; LeDizet and Piperno, 1986). For the DMA1 antibody, the epitope is the C-terminus of the protein, whereas that of the remaining antibody is unknown. No other than wild-type  $\alpha$ -tubulin band was detected with either antibody (data not shown). We repeated the same procedure with cell-body lysates and obtained the same result. Loading as much as 50  $\mu$ g

protein, we were unable to detect any band apart from the one corresponding to wild-type  $\alpha$ -tubulin (Fig. 4); wild-type  $\alpha$ -tubulin was detected with as little as 2  $\mu$ g total protein loaded (data not shown). We, therefore conclude that, in the revertant *tua2-2R1* allele, no detectable  $\alpha_2$ -tubulin is produced. Two other revertant alleles, *tua2-1R1* and *tua2-4R1*, were examined and no base changes other than the original lesion were found in the coding sequence. Both *tua2-1R1* and *tua2-4R1* appear to be intragenic (Table 4). We suspect that these revertants represent mutations in the regions that flank *TUA2*, perhaps altering *TUA2*-regulatory sequences. This conclusion would be consistent with the absence of protein resulting in the reversion of the colchicine-supersensitivity phenotype. However, we cannot exclude the possibility that these revertants contain a mutation in a gene tightly linked to *TUA2*.

#### Treatment with microtubule destabilizers does not rescue the flagellar defects of *uni3-1*

Colchicine supersensitivity suggests that the microtubules in *tua2* strains have decreased stability. We tested the hypothesis that changes in microtubule stability suppress the flagellar phenotypes of *uni3-1* cells in two separate experiments. We asked whether low concentrations of colchicine or oryzalin can phenocopy the suppression of the flagellar defects of *uni3-1* cells. We also tested whether mutations that cause resistance to microtubule-destabilizing drugs obviate the suppression (see following section)? Wild-type and mutant cells ( $2 \times 10^6$  cells



**Fig. 4.** Western blot of proteins from cell bodies of wild-type and *tua2-2R1* cells. Increasing amounts of protein were loaded to detect the predicted 39 kD truncated protein of *tua2-2R1* cells. Lane 1: 7  $\mu$ g of protein from wild-type cells. Lanes 2-5: 7  $\mu$ g, 15  $\mu$ g, 30  $\mu$ g and 50  $\mu$ g of protein from *tua2-2R1* cells. Truncated tubulin protein is not detectable in *tua2-2R1* extracts, only wild-type tubulin.

$\text{ml}^{-1}$ ) were treated for 10 hours with colchicine (0.01 mM to 2.0 mM) and oryzalin (0.1  $\mu$ M to 15  $\mu$ M). This range of drug concentration had little or no effect on viability after 10 hours. There was no change in the number of cells with zero, one and two flagella in *uni3-1* or wild-type cells ( $n=300$ ). Thus, decreasing the stability of the microtubules by pharmacological means does not suppress the flagellar defect of *uni3-1* cells.

#### Suppression is unaffected by mutations that confer resistance to oryzalin

Double mutants were constructed to determine the effect of other tubulin mutations on the suppression of the *uni3-1* allele and on growth on colchicine medium. A mis-sense mutation in  $\beta$ -tubulin (*tub2-1*) confers resistance to colchicine (Lee and Huang, 1990) and results in hyperstabilization of the microtubules (Schibler and Huang, 1991). Mis-sense mutations in  $\alpha_1$ -tubulin (*tua1-1*) and in the *APM1* locus confer resistance to oryzalin, a herbicide that destabilizes microtubules (James et al., 1989; James et al., 1992; Lux and Dutcher, 1991). Double mutants were constructed between *tua2-3* and *tub2-1*, *tua1-1* or *apm1*. In all three double mutants, the colchicine- or oryzalin-resistance phenotypes were epistatic to the colchicine-supersensitivity phenotype (the cells were resistant rather than supersensitive to the drugs) the triple mutants of *tua2-3*; *tua1-1*; *uni3-1* and *tua2-3*; *apm1*; *uni3-1*, the Uni3 flagellar phenotype remained suppressed. The *tub2-1* mutation was not tested in triple mutants with *uni3-1* because the *tua2-3*; *tub2-1* double mutant strain had defects in flagellar assembly.

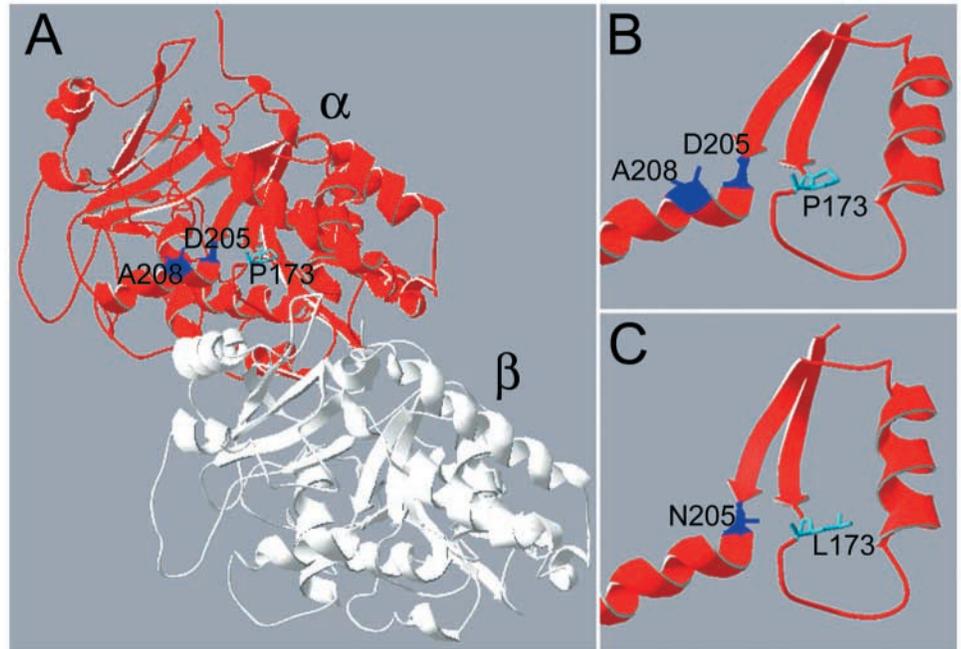
#### Discussion

We have isolated point mutations in *TUA2* that suppress the flagellar assembly phenotype caused by a deletion of  $\delta$ -tubulin, but they fail to suppress the cleavage furrow placement phenotype. We characterized 12 independently isolated suppressors and found that they have one of two amino acid substitutions in  $\alpha_2$ -tubulin: either D205N or A208T. These residues are highly conserved in  $\alpha$ -tubulins across a wide range of species. With two exceptions, they are virtually invariant in the 204  $\alpha$ -tubulin sequences available in GenBank. One exception is the  $\alpha$ -tubulin of the Bermuda land crab that has E instead of D at the position equivalent in *Chlamydomonas* (D205) (GenBank Accession number U92646), the other is the  $\alpha_2$ -tubulin from *Schizosaccharomyces pombe*, which has S at the position equivalent in *Chlamydomonas* (A208) (GenBank Accession number K02842). Alanine scanning mutations in *Saccharomyces cerevisiae* that change the positions equivalent to D205 and E206 to alanine have a cold-sensitive lethal phenotype and benomyl supersensitivity (Richards et al., 2000). The D205N mutation is presumably not deleterious because *Chlamydomonas* has a second gene encoding an identical  $\alpha$ -tubulin protein.

In the crystal structure of  $\alpha$ -tubulin, these residues lie either before or within (D205 and A208, respectively)  $\alpha$ -helix 6 (Fig. 5). The N-terminal end of helix 6 contacts the guanine of the nucleotide (Nogales et al., 1998). This helix is proposed to act as a hinge in  $\beta$ -tubulin that rotates after GTP hydrolysis does not occur (Erickson, 1998; Amos and Löwe, 1999). However, this rotation does not occur in  $\alpha$ -tubulin because GTP hydrolysis. In addition, the position of this helix differs between zinc sheets and microtubules (Li et al., 2002). Zinc ions induce flat sheets of microtubules with antiparallel protofilaments, whereas microtubules have parallel protofilaments. In zinc sheets, helix 6 contacts helix 4 of the adjacent molecule and part of the M loop (Löwe et al., 2001), whereas in microtubules, there is no interaction across the protofilament and a loss of density (Li et al., 2002). Li and co-workers suggest that the loss of density might result from differences in the behavior of helix 6 in  $\alpha$ - and  $\beta$ -tubulin (Li et al., 2002). The difference in the placement of helix 6 in  $\alpha$ - and  $\beta$ -tubulin suggests that this region of  $\alpha$ -tubulin can assume different conformations and is flexible. Structural comparisons of axonemal/basal body microtubules to singlet microtubules would help to address this question. Neither of the amino acid substitutions (D205N and A208T) act by making  $\alpha$ -tubulin more like  $\delta$ -tubulin, which has a E at the position equivalent to D205 and an A at the position equivalent to A208.

What are the roles of  $\delta$ -tubulin and the C tubule? Careful examination of basal bodies from *uni3-1* cells by dual-axis electron tomography (O'Toole et al., 2003) and serial sections reveal that C tubules are present at the distal end of the mutant basal bodies but are missing along the remaining length of the basal bodies. These results show that  $\delta$ -tubulin is not required for initiation of the C tubule but it is probable that  $\delta$ -tubulin is important for either polymerizing or stabilizing the C tubule. Furthermore, full-length C tubules are not required for the assembly because flagella can be assembled on basal bodies that lack a C tubule (Simpson et al., 1997; Preble et al., 2001; Marshall and Rosenbaum, 2003).  $\delta$ -tubulin is needed to stabilize or extend the C tubule. Another important question is whether  $\delta$ -tubulin or the presence of an extended C tubule

**Fig. 5.** The alterations D205N and A208T are close to (D205N) and within (A208T) helix 6 of  $\alpha$ -tubulin. The *Chlamydomonas* amino acid sequence of  $\alpha$ -tubulin (red) and  $\beta$ -tubulin (white) was modeled onto the bovine tubulin crystal structure. (A) Amino acid changes observed in *tua2* alleles (eight blue) and in one revertant allele (blue). (B) Part of the predicted structure, showing helix 6 with A208 and the nearby D205 with respect to the adjacent helix 5 (with P173). (C) The increased length of the side chain in the P173L alteration in revertant *tua2-3RI* has probably major effects on the structure of  $\alpha$ -tubulin. P173 is conserved in most tubulins.

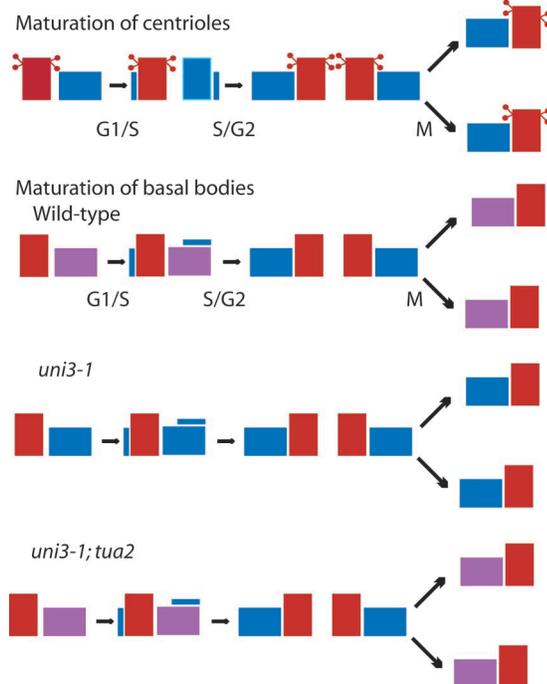


allows a nascent centriole to become a mature basal body that can assemble a flagellum.

Several proteins/epitopes are present on old centrioles and absent from nascent or young centrioles during the G1 and S phase of their first cell cycle in mammalian cells. The acquisition of these proteins is referred to as maturation and occurs throughout the G2 phase of the cell cycle. These proteins include cenexin or outer dense fiber 2 (Lange and Gull, 1995; Piel et al., 2000; Nakagawa et al., 2001), ninein, CEP110 (Ou et al., 2002),  $\epsilon$ -tubulin (Chang and Stearns, 2000),

the dynactin complex (Quintyne and Schoer, 2002) and centriolin (Gromley et al., 2003). Ninein, CEP110 and  $\epsilon$ -tubulin have been localized by electron microscopy to the subdistal appendages located at the distal end of centrioles. The appendages are only present on older centrioles in mammals (Ou et al., 2002; Chang et al., 2003). An older centriole differs functionally from a younger centriole by being able to assemble a primary cilium. This suggests a role for one or more of these proteins and for maturation in promoting the assembly of the cilium. Of those, only  $\epsilon$ -tubulin has been identified to date in *Chlamydomonas* (Dutcher et al., 2002). In addition to the presence of the above proteins, the level of polyglutamylation on  $\alpha$ - and  $\beta$ -tubulin is increased on older centrioles (Bobinnec et al., 1998) and on older basal bodies (Lechtreck and Geimer, 2000). Unlike centrioles, daughter basal bodies must mature early in their first cell cycle in order to assemble flagella or cilia and so for example, all basal bodies in the ciliated epithelium of the trachea have the ODF2 epitope (McKean et al., 2003). Therefore, there is a need for an additional maturation event in basal bodies which we refer to as early maturation and which must occur at M/G1 phase of the cell cycle rather than in G2/M phase, as for centrioles.

The heterogeneous flagellar assembly phenotype in *uni3-1*



**Fig. 6.** The role of  $\delta$ -tubulin in early maturation. Maturation of centrioles occurs at G2/M. Old centrioles are shown in red and young centrioles are shown in blue. Old centrioles are morphologically identifiable by the presence of subdistal appendages. Maturation of basal bodies is hypothesized to occur at two time points in the cell cycle: early maturation occurs at the end of mitosis and a later maturation event takes place at G2/M. Young basal bodies that show early maturation are shown in purple. Old basal bodies are shown in red. Young basal bodies that have not matured are shown in blue. The hypothesis suggests that early maturation fails in *uni3-1* cells as indicated by the lack of purple basal bodies. *tua2* Mutations restore early maturation as indicated by the appearance of purple basal bodies.

cells suggests that  $\delta$ -tubulin might be a signal for early maturation (Fig. 6). Half of the *uni3-1* cells were unable to assemble flagella and the remaining cells assembled one or two flagella. Cells without a flagellum produce one daughter cell without flagella and one with a single flagellum (Dutcher and Trabuco, 1998). There is no difference in the morphology of basal bodies that assemble or do not assemble flagella (O'Toole et al., 2003). A subset of basal bodies in *uni3-1* cells must mature and recruit proteins that are needed for flagellar assembly.

Based on the phenotype of the suppressor mutations, we propose two hypotheses for the role of  $\delta$ -tubulin and the C tubule. The *tua2* suppressors completely suppress the flagellar assembly defect but only partially suppress the C-tubule defect. We found that 13 of the 14 basal bodies have fewer than five extended C tubules, two of which have only a single extended tubule. Our first model proposes that the presence of extended C tubules is sufficient to promote early maturation and that  $\delta$ -tubulin is required for C-tubule stability or extension. In this case, a single extended C tubule must be enough to ensure early maturation, and the *tua2* mutations must act by promoting limited extension of C tubules. The second model suggests that  $\delta$ -tubulin plays two independent roles. First, it is required for stability or extension of the C tubule and, second, it is required for early maturation of the nascent basal body (Fig. 6). In this model, the two functions of  $\delta$ -tubulin are suppressed to different degrees by the  $\alpha$ -tubulin mutations. The mutant  $\alpha$ -tubulin permits all basal bodies to assemble a flagellum but only partially suppresses the ability to assemble the C tubule. The altered  $\alpha$ -tubulin might interact with a protein complex that promotes early maturation more successfully than it does with proteins that affect the stability or extension of the C tubule. Early maturation might involve the recruitment of  $\epsilon$ -tubulin, the intraflagellar transport machinery, which concentrates around the distal region of the basal bodies (Deane et al., 2001), or the Uni1 protein, which is needed for early maturation (Huang et al., 1982). Stability or extension of the C tubule might require unknown microtubule-associated proteins. The *uni3-1; tua2-3R1* double mutant is intriguing in that vegetative cells lack flagella but gametic cells are flagellated. We speculate that different proteins are required for maturation and assembly in gametes and in vegetatively growing cells.

The failure to promote the assembly of the C tubule at the proximal end of the basal body also explains why the cleavage furrow defect (Fig. 3) is not suppressed. The C tubule in *Chlamydomonas* plays a role in the orientation of rootlet microtubules, the extension of centrin fibers of the nucleus/basal-body connector and the attachment of striated fibers between the two basal bodies. Many of these fibers are attached or located at the proximal end of the basal body, which lacks extended C tubules in the suppressed strains. Defects in this fiber system result in defects in cleavage furrow and nuclear placement (reviewed in Dutcher, 2003) (Fig. 6). The cleavage furrow placement is similar to the one observed in *bld2; rgn1* cells, where singlet, doublet and triplet microtubules are present (Preble et al., 2001).

The colchicine-supersensitivity phenotype per se is not necessary for suppression of the *uni3-1* lesion. If destabilized microtubules alone were sufficient, we would have expected colchicine or oryzalin to phenocopy the suppression, but

neither colchicine nor oryzalin suppressed the Uni3 flagellar phenotype. In addition, the triple mutants *tua1-1; tua2-3; uni3-1* and *apm1; tua2-3; uni3-1* conferred resistance to oryzalin, but showed suppression of the Uni3 flagellar phenotype, therefore providing evidence that the altered  $\alpha$ -tubulin is needed for suppression rather than supersensitivity to colchicine.

It is somewhat surprising that none of the isolated suppressors of *uni3-1* show mutations in *TUA1*, another  $\alpha$ -tubulin gene. *TUA1* is tightly linked to *UNI3* on linkage group III (250:0:0 PD:NPD:TT), and the *uni3-1* allele represents a 27 kb deletion. A 7 kb clone containing only the *UNI3* gene rescues the phenotypic defects of *uni3-1* (Dutcher and Trabuco, 1998), which demonstrates that the additional sequences removed in the deletion are apparently not important. However, it is possible that the deletion has altered the DNA structure in the region and thus made the *TUA1* gene less accessible to UV or  $\gamma$ -irradiation mutagenesis. It is also possible that there are slight differences in the expression patterns of the two tubulin genes that favor *TUA2* as a site of mutation for suppression.

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