6 The Isotypes of Tubulin

Distribution and Functional Significance

Richard F. Ludueña
and Asok Banerjee

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SUMMARY

The tubulin molecule is an α/β heterodimer. In most eukaryotes both α- and β-tubulin consist of isotypes encoded by different genes and differing in amino acid sequence. Differences among isotypes are often highly conserved in evolution, suggesting that they have functional significance. The complex isotype families in mammals, *Drosophila* and higher plants have been particularly well studied. Different isotypes often have different cellular and tissue distributions. In addition, purified isotypes display different properties including assembly, GTPase, conformation, dynamics, and ability to interact with anti-tumor drugs. The different cellular, tissue, and species distribution, as well as their primary structures and their *in vitro* properties give clues as to the possible functions of the different isotypes, which will be discussed in this chapter.

Key Words: Tubulin; α-tubulin; β-tubulin; βI; βII; βIII; βIV; βV; βVI; isotypes; anti-tumor drugs; evolution; axonemes; cilia; flagella.

1. INTRODUCTION

Tubulin, the subunit protein of microtubules is an α/β heterodimer *(1,2)*. The full amino acid sequences of α and β were first determined in 1981 and found to be 41% identical *(3,4)*. The existence of tubulin isotypes was confirmed in this same work. The amino acid sequences of the peptides, obtained from pig brain tubulin, showed heterogeneity at various positions, indicating that at least four forms of α and two forms of β were expressed in pig brain, presumably encoded by different genes *(3,4)*. Since that

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time genes for α- and β-tubulin have been sequenced from a large number of eukaryotes. Many of these organisms contain multiple genes for α or β, or both, generally encoding proteins of different amino acid sequence. These different proteins will be referred to as *isotypes* of α or β, meaning proteins encoded by different genes with different amino acid sequences. More recently, other very different forms of tubulin have been discovered, designated as γ, δ, ε, ζ, η, θ, ι, and κ. Still others may be waiting in the wings. These tubulins, together with α and β, are generally grouped together as the tubulin superfamily. Some related proteins have been observed in prokaryotes as well. The tubulin superfamily and the related prokaryotic proteins will be discussed in Chapter 7. In addition to the genetically encoded forms of tubulin, multiple forms of α and β exist, differing in their post-translational modifications. These will be discussed in Chapter 5.

The existence of tubulin isotypes had been predicted long before 1981. In 1967, Behnke and Forer *(5)* had suggested that in view of the different stability of microtubules performing different functions, there must be different forms of tubulin. This proposal was later elaborated into the multitubulin hypothesis, which proposed the existence of such forms, each one responsible for a specific function *(6)*. As will be seen here, the multitubulin hypothesis is fundamentally correct, although not all isotypes can be explained this way, and, in those cases where the hypothesis applies, the functional differences are often far more subtle and complex than originally envisioned. The area of tubulin isotypes has been reviewed before *(7–9)*. Here the concentration will be on discoveries made since 1998.

2. DISTRIBUTION OF TUBULIN ISOTYPES

2.1. Phylogenetic Distribution

The existence of tubulin isotypes has been demonstrated in many organisms (Tables 1–3). It is clear that organisms in every eukaryotic phylum exhibit multiple isotypes of both α- and β-tubulin. This is particularly true for the higher eukaryotes. Among the animals, in every case where multiple isotypes of α and β have been searched for, they have been found. One possible exception is the sea urchin *Lytechinus*, where a single α-tubulin gene was reported *(10)*. However, since this was published, multiple isotypes of α have been found in the sea urchins *Paracentrotus* and *Strongylocentrotus (11,12)*; hence, it is very likely that further investigation will reveal multiple isotypes of α in *Lytechinus* as well. Plants have a similar story. Multiple isotypes of both α- and β-tubulin have been found in every plant that has been investigated. In short, there are no plants or animals that have been found to express either a single α or a single β isotype. Every plant and animal that has been studied expresses multiple isotypes of both α and β.

Protists and fungi, however, are a more complex story. Among the fungi there have been organisms, such as *Candida* or *Histoplasma*, which express only a single α and a single β *(8)*. Others have a single β with multiple α. Interestingly, the converse pattern of a single α with multiple β has not been seen in fungi. Within the different phyla of fungi, all appear to contain species that express multiple α or multiple β isotypes, or both. Expression of a single α or single β is restricted to the ascomycetes and microsporidia (Table 2). In view of the pattern observed with plants and animals, one is tempted to conclude that multicellularity favors the existence of multiple isotypes of α- and β-tubulin.

Table 1
Isotypes of α-, β-, and γ-Tubulin: Animals[a]

Genus	*Phylum/ division*	*Number of isotypes* α	β	γ	*Differences in expression?*	*References*
Homo (human)	Chordate	7	8	2	Yes	*8,76,181,185*
Macaca (Rhesus monkey)	Chordate	ND	≥6	ND	–	*69*
Sus (pig)	Chordate	≥4	≥2	ND	Yes	*3,4*
Bos (cow)	Chordate	ND	≥4	ND	Yes	*64*
Odocoileus (deer)	Chordate	ND	≥2	ND	–	*96*
Canis (dog)	Chordate	ND	≥4	≥1	–	*302,303*
Mus (mouse)	Chordate	6	7	2	–	*8,42,45,304*
Rattus (rat)	Chordate	≥3	≥4	≥1	–	*183,305–311*
Gallus (chicken)	Chordate	5	7	≥1	Yes	*8,63,173,312*
Xenopus (clawed frog)	Chordate	≥2	≥2	≥1	Yes	*8,43,313*
Notothenia (rockcod)	Chordate	≥8	≥4	ND	Yes	*8,288,314*
Chionodraco (icefish)	Chordate	≥4	≥2	ND	–	*288*
Gadus (Atlantic cod)	Chordate	ND	≥4	ND	Yes	*98,293*
Oncorhynchus (salmon)	Chordate	4	ND	ND	–	*8*
Salmo (trout)	Chordate	≥2	ND	ND	–	*8*
Torpedo (electric eel)	Chordate	≥2	ND	ND	Yes	*8*
Danio (zebrafish)	Chordate	2	ND	ND	–	*315*
Ictalurus (catfish)	Chordate	ND	≥2	ND	–	*97*
Mustelus (dogfish shark)	Chordate	ND	≥2	ND	–	*97*
Myxine (hagfish)	Chordate	2	ND	ND	–	*316*
Branchiostoma (lancelet)	Chordate	2	ND	ND	–	*316*
Halocynthia	Tunicata	ND	>2	ND	Yes	*17*
Ciona	Tunicata	3	ND	ND	–	*316*
Oikopleura	Tunicata	10	ND	ND	–	*317*
Paracentrotus (sea urchin)	Echinodermata	4	3	ND	–	*8,11*

(Continued)

Table 1 *(Continued)*

Genus	*Phylum/ division*	*Number of isotypes* α	β	γ	*Differences in expression?*	*References*
Lytechinus (sea urchin)	Echinodermata	1	2	ND	–	*10*
Strongylocentrotus (sea urchin)	Echinodermata	3	≥1	2	–	*12,318*
Gecarcinus (land crab)	Arthropoda	>4	ND	ND	Yes	*319*
Homarus (lobster)	Arthropoda	2	≥1	ND	–	*316*
Heliothis (moth)	Arthropoda	ND	≥2	ND	Yes	*8*
Bombyx (moth)	Arthropoda	≥3	≥4	ND	Yes	*19*
Drosophila (fruit fly)	Arthropoda	4	3	2	Yes	*8,49,320*
Octopus	Mollusca	ND	≥2	ND	Yes	*8*
Aplysia (sea hare)	Mollusca	2	ND	ND	–	*321*
Hirudo (leech)	Annelida	2	ND	ND	–	*322*
Trichostrongylus	Nematoda	ND	≥2	ND	Yes	*209*
Caenorhabditis	Nematoda	4	3	≥1	Yes	*8,21,22, 208,323*
Cyathostomum	Nematoda	ND	≥3	ND	–	*324,325*
Cylicocyclus	Nematoda	ND	≥3	ND	–	*325,326*
Haemonchus	Nematoda	≥1	4	ND	–	*8,207*
Cooperia	Nematoda	ND	≥2	ND	–	*327*
Brugia	Nematoda	ND	≥2	ND	–	*8*
Gyrodactylus	Platyhelminthes	ND	3	ND	–	*328*
Echinococcus (tapeworm)	Platyhelminthes	ND	≥3	ND	–	*329*
Schmidtea	Platyhelminthes	>1	ND	ND	Yes	*20*
Schistosoma	Platyhelminthes	2	ND	ND	–	*8*

[a]The table gives either the actual number of isotypes or else states that there are at least that number. The symbol "≥" as in "≥4" means that there are 4 known isotypes but that there is a reasonable probability of more, based on information from closely related organisms. For more information, *see* ref. *8*, Table 1. For purposes of comparison, the isotypes of γ-tubulin, when known, are included in this table, although γ-tubulin will be discussed further in Chapter 7.

Various patterns of tubulin isotype expression are observed among the protists. Several, such as *Physarum* or *Trichomonas* express multiple isotypes of both α and β; some, such as *Euplotes* express a single α and multiple β; others, such as *Chlamydomonas* and *Plasmodium* have the reverse pattern. *Dictyostelium* expresses only one α and only one β. The widespread occurrence of multiple isotypes among the protists may reflect the complex cellular architecture of some of these organisms.

The knowledge of isotypes of γ-tubulin is still in its infancy, but it is clear that these occur. Multiple γ isotypes have been observed among the animals, plants, and protists, but not among the fungi. γ-tubulin, which is thought to nucleate microtubules, is found

Table 2
Isotypes of α-, β- and γ-Tubulin: Plants and Fungi[a]

Genus	Phylum/ division	*Number of isotypes*			Differences in expression?	References
		α	β	γ		
Plants						
Daucus (carrot)	Angiosperm	≥1	>4	ND	Yes	*24*
Pisum (pea)	Angiosperm	ND	3	ND	–	*8*
Glycine (soybean)	Angiosperm	ND	3	ND	Yes	*8,47*
Solanum (potato)	Angiosperm	ND	≥2	ND	–	*8*
Eucalyptus	Angiosperm	>1	ND	ND	–	*8*
Zinnia	Angiosperm	ND	≥3	ND	Yes	*8*
Prunus (plum)	Angiosperm	>1	ND	ND	Yes	*8*
Oryza (rice)	Angiosperm	3	3	≥1	Yes	*8,330,331*
Triticum (wheat)	Angiosperm	>1 6	ND	Yes	–	*25,332*
Arabidopsis (cress)	Angiosperm	4	8	2	Yes	*8,333*
Nicotiana (tobacco)	Angiosperm	2	5	≥1	Yes	*334–338*
Hordeum (barley)	Angiosperm	5	≥3	≥1	Yes	*28,339*
Gossypium (cotton)	Angiosperm	≥5	≥6	ND	–	*26,340*
Lupinus (lupine)	Angiosperm	ND	≥2	1	Yes	*8,341*
Populus (aspen)	Angiosperm	3	ND	ND	–	*342,343*
Cosmos (sunflower)	Angiosperm	≥2	ND	ND	Yes	*44*
Zea (corn)	Angiosperm	≥6	8	2	Yes	*8,344,345*
Eleusine (goosegrass)	Angiosperm	≥3	≥4	ND	–	*346,347*
Miscanthus	Angiosperm	8	ND	ND	–	*348*
Anemia (fern)	Angiosperm	2	2	1	–	*349,350*
Physcomitrella (moss)	Bryophyta	2	5	1	–	*351–353*
Fungi						
Histoplasma	Ascomycota	1	1	ND	–	*8*
Aspergillus	Ascomycota	2	2	1	Yes	*8*
Colletotrichum	Ascomycota	2	2	ND	Yes	*8,33,354*
Candida	Ascomycota	1	1	1	–	*8,355,356*
Neurospora	Ascomycota	2	1	1	Yes	*8,35,357*
Trichoderma	Ascomycota	ND	≥3	ND	–	*8,358*
Hypocrea	Ascomycota	ND	2	ND	–	*8*
Paracoccidioides	Ascomycota	2	ND	ND	Yes	*36*
Botryotinia	Ascomycota	ND	1	ND	–	*359*
Erysiphe (grass mildew)	Ascomycota	ND	1	ND	–	*8*
Epichloe	Ascomycota	ND	1	ND	–	*8*
Saccharomyces	Ascomycota	2	1	1	No	*8,213*
Schizosaccharomyces	Ascomycota	2	1	1	No	*8,313*
Pneumocystis	Ascomycota	1	1	ND	–	*8,360*
Geotrichum	Ascomycota	ND	2	ND	–	*8*
Conidiobolus	Zygomycota	2	≥1	ND	–	*361,362*
Rhizopus	Zygomycota	3	3	ND	–	*361,362*
Basidiobolus	Zygomycota	2	2	ND	–	*362*

(Continued)

Table 2 *(Continued)*

Genus	*Phylum/ division*	*Number of isotypes* α	β	γ	*Differences in expression?*	*References*
Spiromyces	Zygomycota	ND	2	ND	–	*362*
Mortierella	Zygomycota	ND	2	ND	–	*362*
Powellomyces	Chytridiomycota	3	ND	ND	–	*361,362*
Allomyces	Chytridiomycota	ND	2	ND	–	*363*
Spizellomyces	Chytridiomycota	ND	2	ND	–	*361*
Harpochytrium	Chytridiomycota	ND	2	ND	–	*361*
Glomus	Glomeromycota	ND	2	ND	–	*364*
Suillus	Basidiomycota	ND	2	ND	–	*365*
Cryptococcus	Basidiomycota	ND	2	ND	No	*366*
Encephalitozoon	Microsporidia	1	1	1	–	*8,367,368*

[a]*See* explanation under Table 1.
For more information, *see* ref. *8,* Table 2.

Table 3
Isotypes of α-, β-, and γ-Tubulin: Protists[a]

Genus	*Phylum/ division*	*Number of isotypes* α	β	γ	*Differences in expression?*	*References*
Cryptosporidium	Apicomplexa	ND	1	1	–	*8,369,370*
Toxoplasma	Apicomplexa	3	3	ND	–	*8,38*
Babesia	Apicomplexa	ND	1	ND	–	*8*
Plasmodium	Apicomplexa	2	1	1	–	*8,371*
Eimeria	Apicomplexa	ND	1	ND	–	*8*
Physarum (slime mold)	Mycetozoa	3	4	2	Yes	*8,372*
Chloromonas (snow alga)	Chlorophyta	2	ND	ND	–	*373*
Chlamydomonas	Chlorophyta	2	1	1	–	*8,374*
Polytomella	Chlorophyta	ND	2	ND	–	*8*
Volvox	Chlorophyta	ND	2	ND	–	*8,375*
Paramecium	Ciliophora	2	1	1	–	*8,376*
Tetrahymena	Ciliophora	1	2	1	–	*8,377*
Stylonichia	Ciliophora	2	1	ND	–	*8*
Euplotes	Ciliophora	1	4	2	–	*378,379*
Moneuplotes	Ciliophora	3	≥1	2	–	*8,380,381*
Histriculus	Ciliophora	1	ND	ND	–	*382*
Moneuplotes	Ciliophora	5	ND	ND	–	*381*
Tintinnopsis	Ciliophora	2	ND	ND	–	*383*
Strobilidium	Ciliophora	3	ND	ND	–	*383*
Metacylis	Ciliophora	2	ND	ND	–	*383*
Laboea	Ciliophora	3	ND	ND	–	*383*
Strombidinopsis	Ciliophora	6	ND	ND	–	*383*
Favella	Ciliophora	2	ND	ND	–	*383*
Opisthonecta	Ciliophora	2	ND	ND	–	*384*

(Continued)

Table 3 *(Continued)*

Genus	*Phylum/ division*	*Number of isotypes* α	β	γ	*Differences in expression?*	*References*
Halteria	Ciliophora	6	ND	ND	–	*381*
Metopus	Ciliophora	3	ND	ND	–	*381*
Heliophrya	Ciliophora	3	ND	ND	–	*381*
Nyctotherus	Ciliophora	2	ND	ND	–	*381*
Dictyostelium	Acrasiomycota	1	1	1	–	*8,38*
Ectocarpus	Phaeophyta	ND	2	ND	–	*8*
Chondrus	Rhodophyta	ND	2	ND	–	*8*
Achlya	Oomycota	ND	1	ND	–	*8*
Amphidinium (dinoflagellate)	Dynophyceae	2	ND	ND	–	*386*
Reticulomyxa	Rhizopoda	2	2	1	–	*8,387*
Naegleria	Heterolobosea	≥4	>1	ND	Yes	*8,37*
Leishmania	Euglenozoa	≥1	2	1	Yes	*8,388,389*
Trypanosoma	Euglenozoa	1	1	1	–	*390,391*
Trichomonas	Parabasalidea	2	≥3	ND	Yes	*8,392*
Tritrichomonas	Parabasalidea	≥2	ND	ND	–	*393*
Trichonympha	Parabasalidea	2	≥1	ND	–	*394*
Hypotrichomonas	Parabasalidea	2	3	ND	–	*395*
Monocercomonas	Parabasalidea	ND	2	ND	–	*396*
Pelvetia (brown alga)	Phaeophyceae	≥2	ND	ND	–	*397*
Bigelowiella	Cercozoa	3	3	ND	–	*398*
Pyrsonympha	Oxymonadida	2	ND	ND	–	*394*
Streblomastix	Oxymonadida	5	1	ND	–	*399*

[a]*See* explanation under Table 1.
For more information, *see* ref. *8*, Table 3.

in centrosomes as well as other microtubule organelles. It is conceivable that fungi, which lack centrosomes, may not require more than a single isotype of γ-tubulin.

2.2. Tissue, Cellular, and Subcellular Distribution

What functions do isotypes serve? Why have the differences among isotypes in groups such as the vertebrates been so widely conserved? The fact of this conservation argues that the differences must matter, but it does not prove it. One could argue that all isotypes are completely interchangeable functionally and that there is a space of certain amino acid sequences that are compatible with function. Evolution has randomly filled at least part of this space. In other words, conceivably, mammalian βI, for example, could accept certain mutations and still assemble into a microtubule that would perform all microtubule-mediated functions; mammalian βIII could do likewise. However, βI could not mutate into βIII, because the intermediate forms would not be viable. This would help to account for the preservation of isotype differences in evolution. What about the fact that isotypes often differ in their tissue distribution? One could further argue that when a certain tissue differentiates, a cassette of genes is expressed that happens to include one particular isotype and not another. The fact that certain isotypes

Table 4
Tubulin Isotypes in the Inner Ear of the Gerbil[a]

Cell	β*I*	β*II*	β*III*	β*IV*
Cochlea (adult)				
Outer hair cell	+	–	–	+
Inner hair cell	+	+	–	–
Outer pillar cell	–	+	–	+
Inner pillar cell	–	+	–	+
Deiters cell	+	+	–	+
Schwann cell	+	?	?	?
Neurons	+	+	+	?
Afferent dendrites	–	–	–	–
Cochlea (developing)				
Outer hair cell	+	+	–	+
Inner hair cell	+	+	–	+
Outer pillar cell	+	+	–	+
Inner pillar cell	+	+	–	+
Deiters cell	+	+	–	+
Afferent dendrites	+	+	+	–
Vestibular organ (adult)				
Type I hair cell	+	–	–	+
Type II hair cell	+	–	–	+
Supporting cell	+	+	–	+
Schwann cell	+	?	?	?
Neurons				
Axons, soma	+	+	+	–
Dendrites	+	+	+	–
Calyx	–	–	+	–

Source: Adapted from refs. *13–15*.
[a]Absence of signal could indicate either that the isotype as not present in the tissue or that extensive post-translational modification made it undetectable to the antibody.

have their expression regulated by particular factors is consistent with this model, as will be discussed later. The result would be tissues expressing different isotypes. Each isotype would be participating in certain generic processes such as mitosis as well as tissue-specific processes such as secretion in the liver or axonemal motility in tracheal epithelia. However, by this model the isotypes would be interchangeable. In other words, if the liver isotype were to be expressed in the tracheal epithelia and not in the liver and conversely for the tracheal isotype, processes such as secretion and axonemal motility would not be compromised. The ideal way to prove that the structural differences among isotypes have functional significance is to demonstrate that the isotypes are not functionally interchangeable. As will be seen later, this has been done in a few cases. In addition, there is a great deal of other evidence that supports the hypothesis that isotype differences are functionally significant.

As just discussed, the differences in tissue distribution among tubulin isotypes do not constitute definitive evidence that the isotype differences are functionally significant. Nevertheless, in many cases, the distribution of isotypes among tissues and even among different cell types in the same tissue is extremely complex. Table 4 shows the distribution

Table 5
Distribution of Tubulin Isotypes in Maize[a]

Cell/tissue	β1	β2	α1	α3	α5
Seedling root tip cells	+	–	+	+	–
Seedling leaf epidermis	+?	–	+	+	ND
Male meiocytes	+	+	+	+	ND
Pollen tubes					
Axial microtubules	–	–	+	–	–
Microtubules associated with either vegetative nuclei or sperm cells	–	–	+	+?	+?

[a]*Source:* Ref. *400.*

of isotypes in different cells of the inner ear. It is striking that adjacent cells can have different isotype compositions, even in cells that perform similar although not identical functions such as the inner and outer hair cells of the cochlea *(13)*. In addition, the pattern of isotypes changes during development. For example, the inner and outer hair cells express the same set of β isotypes (βI, βII, and βIV) in early development and then the outer hair cells stop making βII, whereas the inner hair cells stop expressing βIV *(14)*. It is difficult to ascribe this complexity to different cassettes of genes.

In certain cases, isotype distributions appear to differ within the same cell. For example, some of the neurons of the gerbil vestibular organ have a portion, called the calyx, which is like a cup enveloping the adjacent hair cell. Although the rest of the neuron contains βI, βII, and βIII, only βIII occurs in the calyx (Table 4) *(15)*. If the cell is unable to discriminate among the isotypes, how is it able to arrange that βI and βII, but not βIII, be restricted from entering the calyx? If the cell is able to distinguish the isotypes from each other, then it is easy to imagine that the different isotypes can perform different functions. Nevertheless, there is still one way out of the dilemma posed by the vestibular neurons, a way that would still allow us to maintain the functional interchangeability of isotypes. A highly elaborate series of temporally regulated cassettes of genes can be posited, such that only βIII is expressed when the calyx is forming, whereas all three isotypes are expressed before that time.

Isotype distributions are also complex in other animals. This complexity has been seen in the frog *Rana (16)*, the tunicate *Halocynthia (17)*, sea urchins *(18)*, the fruit fly *Drosophila*, the moths *Heliothis* and *Bombyx (19)*, the mollusc *Patella*, the nematode *Brugia,* and even the platyhelminth *Schmidtea (20)* (reviewed in refs. *8,9*).

An intriguing example of isotype distributions has been observed in the nematode *Caenorhabditis elegans*. These organisms contain touch receptor neurons whose microtubules are made up of 15 protofilaments, instead of the more usual 11 protofilaments as are the other microtubules of *C. elegans*. The tubulin dimers that constitute these "giant" microtubules consist of a unique α and a unique β isotype *(21,22)*. As will be argued later for the mammalian βVI isotype, it is possible that microtubules of unique morphology require unique tubulin isotypes. Of the other isotypes in *C. elegans*, some interchangeability has been observed, but one β isotype is required for centrosomes to be stable *(23)*.

Isotype distributions are complex in plants as well as shown for maize in Table 5. Similar complex tissue distributions of plant tubulin isotypes have been found in *Arabidopsis*, soybean, carrot *(24)*, wheat *(25)*, tobacco, and plum (reviewed in refs. *8,9*).

More recently, complex distributions of isotypes have been reported in cotton *(26)*, rice *(27)*, and barley *(28)*. Interestingly, one of the rice β-tubulin genes encodes three different mRNA species, thereby creating even more isotypes *(29)*, a rare example of tubulin isotypes arising by alternative splicing.

The relative levels of plant tubulin isotypes appear to be controlled by hormones such as gibberellin *(27)* as well as by factors that selectively degrade the mRNAs for particular isotypes *(30)*. The story of barley is probably typical. Schröder et al. *(28)* did not attempt to study the entire set of tubulin isotypes, but only the five α isotypes in the leaf. They found that α3 was probably constitutive, being expressed at each stage of leaf development. α2 and α4 were found largely in meristematic cells, declining during later stages of differentiation (α2 declined more rapidly than α4). α1 and α5, however, appeared very transiently only in the rapidly growing cells; these cells contain microtubule bundles that determine the later morphology of the leaf cells *(31)*. This work teaches a valuable lesson in indicating that an important tubulin isotype can appear, do its job, and then quickly disappear, and hence may escape detection in experiments. Mutants of two specific isotypes of *Arabidopsis* α-tubulin altered the growth pattern of the hypocotyls and the pattern of microtubules in the root *(32)*, as one would expect given the different tissue distributions of the *Arabidposis* isotypes.

Fungi are simpler than plants and animals. Nevertheless, differences in tubulin isotype expression have been observed in *Colletotrichum (33)*, *Aspergillus*, *Fusarium (34)*, *Neurospora (35)*, and *Paracoccidioides (36)* (reviewed in refs. *8,9*). In some cases, these organisms have one isotype that is high during the vegetative phase and one during conidiation *(33,34)*.

Differences in expression are occasionally seen in protists, even though they are single-celled organisms. This is sometimes the case in different stages of the life cycle and has been observed in *Plasmodium*, *Leishmania*, *Physarum*, *Naegleria (37)*, and *Toxoplasma (38)* (reviewed in ref. *8*). In the case of *Physarum*, for example, of the three stages of its life cycle—amoeba, plasmodium, and flagellate—a different β isotype predominates at each stage, whereas the α isotypes differ as well, but not so strikingly *(39,40)*.

One oddity of tubulin distribution is that when an organism with multiple isotypes has an α or a β isotype of unusual sequence that isotype is often associated with the reproductive system. In *Drosophila*, the α4 isotype is only 67% identical to the other three α isotypes; it is uniquely expressed in the oocyte and the early embryo *(41)*. The mouse αTT1 isotype, sharing about 70% identity to the other α, is expressed only in the testis *(42)*. *Xenopus* has an unusual α expressed in the ovary *(43)*. The platyhelminth *Schmidtea* has a highly divergent α expressed only in the testis *(20)*. Sunflower pollen has a unique α-tubulin, much more basic than other α. It is even thought to have a different tertiary structure with an altered H1/B2 loop facing into the interior of the microtubule *(44)*. The fungus *Colletotrichum* has a divergent β expressed only in its conidia *(33)*. The protist *Naegleria* expresses three α isotypes, one of which is only 61.9% identical to the other two α; the unusual α is not expressed in the flagellate, but only in the dividing amoeba, where it is found in the spindle *(37)*. It is hard to account for these divergent isotypes being restricted to the reproductive tissues. If the divergent isotype in one organism had a striking resemblance to the corresponding isotype in another, one could argue that the isotypes shared a particular structural feature that is necessary to perform a certain function related to reproduction; formation of the meiotic spindle would be a tempting candidate. However, the divergent isotypes not only do not resemble each other, they can occur in either male or female reproductive organs. As most of

these divergent isotypes are α, it may be that there is a particular function carried out by the β-subunit in, say, meiosis and that this function does not involve α at all. Perhaps the α- and β-subunits in reproductive cells are expressed in the same cassette of genes. If only β is performing a stringent function, one could then argue that this situation leaves α free to diverge significantly in the course of evolution.

Not all of the highly divergent isotypes occur in reproductive tissues, however. Mammals and birds have a very divergent β isotype whose expression is restricted to haematopoietic tissues, including erythrocytes and platelets *(45,46)*. This will be discussed later in more detail. The soybean produces a divergent β isotype; low levels of this isotype are expressed in the cotyledon, and high levels in the hypocotyl, when the soybean is grown in the absence of light *(47)*. It may be that an isotype that performs only a single function is more likely to diverge in the course of evolution than one that is involved in a large number of processes.

3. FUNCTIONS OF TUBULIN ISOTYPES

3.1. Tubulin Isotypes in Drosophila

The most unambiguous demonstration of isotype-specific functions comes from a series of experiments done in the fruit fly *Drosophila*. Early experiments showed that mutation of the testis-specific β2 isotype caused inability to form axonemes of normal morphology and function *(48–51)*. Similar results were obtained when β2 was replaced by the divergent β3 isotype; meiosis was blocked as well *(52)*. It is interesting that loss of β2 blocks meiosis but not mitosis. This observation may be connected with the fact that in *Drosophila*, the meiotic spindle is surrounded by a membranous structure *(53)*. Conceivably, the β2 isotype may play a role in interactions of the meiotic spindle with that membrane.

Alterations in the β3 isotype also result in specific changes. This isotype appears for a short time during embryogenesis. Mutants of β3 have poor sensory perception. Microtubules in the chordotonal sensory organ are more highly crosslinked in the mutant than in the wild-type. The authors suggest that increased crosslinking may inhibit flexibility during development leading to impaired function later on *(54)*. Perhaps β3 has a smaller propensity to form crosslinks. In addition, β3 expression correlates with muscle development whereas β1 expression is induced by attachment to the epidermis *(55)*.

The α-tubulin isotypes of *Drosophila* also appear to have specific functions. Komma and Endow *(56)* showed that the α67C isotype binds to the motor protein Ncd whereas the α84B isotype does not. Mutations in α67C alter meiosis I and decrease the accuracy of chromosome segregation *(57)*. Hutchens et al. *(58)* found that replacement of the α84B with the very similar (98% identical) α85E led to synthesis of abnormal axonemes, often lacking the central pair microtubules as well as the outer singlet, or accessory, microtubules, characteristic of insect sperm flagella.

3.2. Mammalian Tubulin Isotypes: the β-Isotypes

In addition to *Drosophila*, a good deal is now known, or at least hypothesized, about the functional assignments of the β-tubulin isotypes in mammals. This will be reviewed later. As will be seen, the functional significance of some of the isotypes is fairly certain, others are speculative, and some are completely unknown.

Table 6
Vertebrate β-Tubulin Isotypes[a]

Designation	*Species*	*C-terminal sequence*	*Distribution*
Class Ia	Human	YQDATAEEEEDFGEEAEEEA	Widespread
	Mouse	YQDATAEEEEDFGEEAEEEA	
	Rat	YQDATAEEEEDFGEEAEEEA	
	Chicken	YQDATAEEEEDFGEEAEEEA	
Class Ib	Human	YQDATAEEEEDFGEEAEEEA	Retina
Class II	Human	YQDATADEQGEFEEEEGEDEA	Brain, muscle, and so on
	Mouse	YQDATADEQGEFEEEEGEDEA	
	Rat	YQDATADEQGEFEEEEGEDEA	
	Chicken	YQDATADEQGEFEEEGEDEA	
	Gadus	YQDATADEEGEFDEEAEEDG	
	Notothenia	YQDATAEEEGEFEEEGEYEDGA	
Class III	Human	YQDATAEEEGEMYEDDEEESEAQGPK	Neurons, Sertoli, and so on
	Rat	YQDATAEEEGEMYEDDDEESERQGPK	
	Chicken	YQDATAEEEGEMYEDDEEESEQGAK	
	Xenopus	YQDATAEEEGEMYEDDEEESEGQGK	
	Gadus	YQDATAEEEENFDEEADEEIA	
Class IVa	Human	YQDATAEQGEFEEEAEEEVA	Brain
	Mouse	YQDATAEEGEFEEEAEEEVA	
Class IVb	Human	YQDATAEEEGEFEEEAEEEVA	Widespread, esp. in ciliated tissues, sperm
	Mouse	YQDATAEEGEFEEEAEEEVA	
	Rat	YQDATAEEGEFEEEAEEEVA	
	Chicken	YQDATAEEEGEFEEEAEEEAE	
	Gadus	YQDATAEEEGEFEEEGEEELA	
	Notothenia	YQDATAEEEGEFEEEGEEDLA	
Class V	Human	YQDATANDGEEAFEDEEEEIDG	Unknown
	Mouse	YQDATVNDGEEAFEDEDEEEINE	
	Chicken	YQEATANDGFEAFEDDEEEINE	
	Xenopus	YQEATANDEEEAFEEDEEEVNE	
Class VI	Human	FQDAKAVLEEDEEVTEEAE MEPEDKGH	Platelets, bone marrow
	Mouse	FQDVRAGLEDSEEDAEEAEV EAEDKDH	
	Chicken	YQDATADVEEAEASPEKET	
Class VII	Human	YQDATAEGEGV	Unknown
Unclassified	*Notothenia*	YQDATADEMGEYEEDEIEDE EEVRHDVRH	

[a]*Source:* From refs. *45,63,67,69,181,182,217,307–310,401–403*.
The chicken has two forms of βII, differing from each other at 2 out of 445 positions *(63)*.

3.2.1. βI

The βI isotype appears to be the most widespread among mammalian tissues (Table 6). It has been seen in almost every tissue that has been examined *(8,59)*. It is also found in many avian tissues *(60)*. It is highly conserved in evolution: although the avian and

mammalian lines diverged 310 million years ago (mya) *(61)*, chicken and mouse βI are identical in all 444 residues *(45,62,63)*. The relative amounts of βI in different tissues are very variable. In cow brains βI constitutes about 3–4% of the total β-tubulin *(64)*; by contrast, in the thymus βI appears to be the major β isotype *(60)*. In fact, thymus tubulin was used as the positive control in the selection of the monoclonal antibody to βI *(59)*. However, βI is probably not a constitutive tubulin. In the gerbil cochlea, for example, βI is expressed in hair cells but not in pillar cells *(13)*. Also, follicle-stimulating hormone induces expression of βI in rat granulosa cells *(65)*, suggesting that it may be performing a specific function, although one could argue that the hormone is merely stimulating cell proliferation, which would in turn require microtubule assembly. βI is clearly not constitutive in zebrafish, where its expression is limited to the nervous system throughout development, and in the adult brain is restricted to the regions where proliferation is occurring *(66)*. Higher primates appear to have two very similar forms of βI, but they are unlikely to differ in function *(67–69)*. Mice and chickens have only a single βI.

What might be the role of βI? Narishige et al. *(70)* found that cardiac hypertrophy is accompanied by increased βI and βII. They speculated that βI may play a role in increasing microtubule stability. There is some evidence indicating specific roles for βI. First, it is found in a variety of mammalian cilia, including those of nasal epithelia, tracheal epithelia, vestibular epithelia, and oviduct epithelia *(15,71,72)*. Traces of βI have been observed in mouse sperm as well *(9)*. As will be discussed further later, the major constituent of ciliary and flagellar axonemes is βIV, which has the signal sequence (EGEFEEE) proposed by Raff et al. *(73)* to be a requirement for a β-tubulin to be incorporated into axonemes. However, although βI lacks that signal sequence, it is conceivable that the signal sequence requirement does not apply to all of the microtubules in the axoneme. Certainly, the structure of axonemal microtubules is sufficiently complicated that it is easy to visualize that there are more than enough functions to be distributed among two isotypes. For example, one could speculate that βI could form one or both of the central pair microtubules or the B-tubules of the outer doublets.

The clearest evidence for a specific function for βI was obtained by Lezama et al. *(74)* MDCK cells, βI was relatively depleted in the cortical regions of MDCK cells, an area that is rich in actin filaments. They also observed that overexpression of βI tubulin in MDCK cells and incorporation of exogenous βI tubulin into microtubules interferes with adhesion and spreading. They suggest that βI may interfere with the actin–tubulin interaction. Very recently another possible function for βI was suggested. Yanagida et al *(75)* found that human fibrillarin forms a complex with the α3 and βI isotypes of tubulin. Fibrillarin is involved in ribosome assembly and processing of rRNA *(75)*. The specific role of tubulin in this process is unknown.

3.2.2. βII

The brain is the source of the tubulin used in the vast majority of experimentation in vitro. As βII constitutes 58% of the total β-tubulin in bovine brain *(64)*, one could say that βII is the best studied of the tubulin isotypes. For this reason, it is highly ironic that so little is known about βII specific function. However, as βII is highly conserved in evolution, it probably has a particular role to play. βII has a considerably more restricted distribution than does βI. βII is prominent in the brain, where it is expressed in both neurons and glia. βII is also found in skeletal and smooth muscle and in connective tissue *(76)*. It is found in the breast, adrenal, and testis as well *(77,78)*. In other tissues where βII occurs, it is more likely to be restricted to a single cell type than is βI. For example,

in the skin, where βI is expressed in each of the three layers of the stratum malpighii, βII is concentrated in only one of these layers, the stratum granulosum *(59)*.

βII is more widespread in early development. In fetal rats, not only does βII occur in muscles, nerves, and connective tissue but also in the retina, chondrocytes, and endothelial cells *(77)*. Not surprisingly, βII also is found in neural stem cells *(79)*. Unlike βI and βIV, βII is generally not associated with axonemal microtubules except for those of the cilia of olfactory epithelia *(71)*. The significance of this finding is uncertain. An immunogold electron microscopic study of axonemes in retinal and tracheal cilia showed that βII was present near the axonemes but did not form part of their microtubules, unlike βIV, which was clearly incorporated into the axonemal microtubules *(80)*. βII, thus, is probably not adapted to function in axonemal microtubules. One study in HeLa interphase cells found that βII was concentrated in the perinuclear region and the periphery of these cells. Cold treatment (which causes microtubules to break up) resulted in βII being associated with the centrosome and the cell periphery; nocodazole treatment had the same effect *(81)*. This finding raises the possibility that βII may play a role in anchoring microtubules to the centrosome and the cell periphery.

A highly unusual property of βII has recently been discovered. Ranganathan et al. *(82)* observed that βII, but not βI, βIII, or βIV, occurred in the cell nuclei of prostate tumors and benign prostate hyperplasia. A later study showed that βII was present in the nuclei of cultured rat kidney mesangial cells in interphase in nonmicrotubule form *(83)*. This will be discussed later on. The possibility will be raised that βII may play a role in organizing the nuclear membrane during mitosis. Even if βII has a function involving the cell nucleus and mitosis, however, this does not seem sufficient to explain its very high concentration in neurons, which undergo little or no cell division and, which appear to have a very high ratio of cytoplasm to nucleus. A similar argument would apply to muscles, which are also rich in βII *(76)*, although it is perhaps relevant that in muscle, microtubules are nucleated by the nuclear membrane rather than by the centrosome *(84)*. In nerves and muscles, βII probably has other functions, totally unrelated to mitosis, but what these functions may be is a complete mystery.

3.2.3. βIII

3.2.3.1. Unusual Characteristics of βIII. The βIII isotype has six distinguishing characteristics, each of which is probably relevant to developing an understanding of its functional significance.

1. β*III is highly conserved in evolution*. As is the case with βI, there are only two differences in the amino acid sequences of chicken and human βIII *(85,86)*.
2. β*III has a highly unusual distribution of cysteines*. All the vertebrate β isotypes have cysteines at positions 12, 127, 129, 201, 211, 303, and 354. The more widely distributed β isotypes—βI, βII, and βIV—also have a cysteine at position 239. βIII lacks this cysteine but has a cysteine at position 124 instead, where βI, βII, and βIV have a serine. The significance of these cysteines will be discussed later.
3. β*III has an extremely narrow distribution in normal adult tissues*. It is the most abundant in the brain, where it is found only in neurons and not in glial cells (by contrast, βII is found in both) *(87)*. Its absence from glial cells has made βIII a useful marker for neuronal differentiation *(88,89)*. βIII synthesis can be induced by factors such as androgens *(90)*, STEF *(91)*, and nerve growth factor *(92)*. The latter, when combined with retinoic acid, can cause human umbilical cord blood cells to synthesize βIII as well as

other neuronal proteins *(92)*. βIII also occurs in Sertoli cells and, in small amounts, in the vestibular organ, the nasal epithelia, and the colon *(93)*. In other adult tissues that have been examined, βIII appears to be absent. However, βIII is found in a large number of cancers and is also widespread in some developing tissues.

4. *When tubulin is reduced and carboxymethylated,* β*III has a unique electrophoretic mobility on polyacrylamide gels in the system of Laemmli (94,95)*. This feature has made it easy to measure its levels in the brains of different vertebrates. βIII accounts for 25% of the total β-tubulin in the brains of cows and 20% in deer brains *(96)*. The fact that, unlike the more abundant βII, βIII occurs only in neurons and not in glial cells, however, suggests that the relative amount of βIII in neurons must be very high indeed. This is consistent with the observation that βIII accounts for 25.7 ± 0.7% of the total β in bovine cerebral gray matter and only 20.7 ± 0.5% in white matter, the latter being enriched in glial cells *(135)*. In the brains of chickens, dogfish shark, and catfish, βIII accounts, respectively, for 14%, 8–17% and 10%, of the total β *(96,97)*. Interestingly, in a cold-adapted fish, the Atlantic cod *Gadus morhua*, βIII accounts for 30% of the total β tubulin *(98)*. However, in other cold-adapted fishes, the Antarctic cod *Notothenia* and the Antarctic icefish *Chaenocephalus*, βIII accounts for 8–12% and 4%, respectively *(97)*.
5. β*III is phosphorylated at a serine near the C-terminus (99)*. Except for βVI, the other vertebrate β isotypes have no serines in this region and thus cannot be phosphorylated here.
6. *The dynamic behavior* in vitro *of microtubules made of the* αβ*III dimer is higher than that of microtubules made of either the* αβ*II or* αβ*IV dimers (100)*.

3.2.3.2. βIII is likely to be less sensitive to reactive oxygen species (ROS) and free radicals. Let these observations be put together to see if they point to a specific functional role for βIII. The unusual cysteine distribution is a good place to begin. It has long been known that microtubule assembly in vitro and in vivo is exquisitely sensitive to sulfhydryl-oxidizing agents *(101)*. Cys239 in β is very reactive and its oxidation inhibits assembly *(102,103)*. In other words, a tubulin molecule oxidized at cys239 cannot assemble onto a microtubule *(104)*. βIII lacks cys239 and has ser239 instead; βV and βVI also have ser239. It has been shown that the αβIII and αβVI dimers are significantly less reactive with alkylating agents than are the other isotypes and that the polymerization of αβVI is less inhibited by alkylation *(96,105)*. It must be emphasized that the presence of a serine at position 239 is highly unusual among tubulins. Outside of βIII, βV, and βVI, every other animal β-tubulin contains cys239 *(8)*. Also, almost every plant and protist β-tubulin has a cysteine at either position 239 or 238 or both. Fungal β-tubulins are virtually the only ones without a cysteine in this area. βIII also contains a cysteine at position 124 and this is even more unusual. Except for βV and avian βVI, there is no β-tubulin in any eukaryote with a cysteine at position 124. The fact that cys124 and ser239 are both highly conserved in the evolution of βIII and highly unusual in the universe of β-tubulins strongly indicates that these particular residues must play a major role in the function of βIII.

It is probably not a coincidence that cys124 is very close to the highly conserved cys127 and cys129. Most β-tubulins have cysteines at these positions. These three cysteines (124, 127, and 129) constitute a cysteine cluster. A cysteine cluster of identical topography occurs in Von Willebrand's protein, a giant serum protein that promotes blood coagulation. In Von Willebrand's protein, the cysteine cluster is the site of interchain disulfide bonds *(106)*. Von Willebrand's protein also contains sets of vicinal cysteines (with two residues between the cysteines)—as with cys124 and cys127 in

βIII—that appear to undergo sulfhydryl-disulfide interchanges during polymerization *(107)*. It is conceivable that such an interchange occurs when the αβIII dimer polymerizes. On the other hand, it is possible that an intrachain disulfide forms in response to oxidation; This possibility will be pursued later. Although it is generally thought that disulfide bridges in proteins cannot form in the cytosol, recent evidence indicates that the SV40 protein Vp1 forms transitory intrachain and interchain disulfides whereas folding and oligomerizing in the cytoplasm. The mature virus has no disulfides of any kind *(108)*.

The absence of a cysteine at position 239 in βIII is probably very telling, especially when one considers the effects of nitric oxide (NO) and ROS on microtubules. ROS are generated by mitochondria and can also be found in the diet. These species can react with sulfhydryl groups. In view of the overall high reactivity of the sulfhydryl group of cys239, it is easy to imagine it reacting with ROS. In addition, certain tissues synthesize NO, which is itself a free radical capable of reacting with sulfhydryl groups. One ROS, O_2^- (superoxide anion), reacts with NO to make peroxynitrite ($ONOO^-$) *(109)*. $ONOO^-$ in turn reacts with tubulin to form disulfide bridges between the α- and β-subunits *(109,110)*. Cys239, which is close to the α/β interface *(111)*, probably participates in this disulfide, which inhibits microtubule assembly *(112)*. Lacking this cysteine, the assembly of βIII would not be inhibited. Thus, βIII is likely to be less sensitive to free radicals.

3.2.3.3. βIII is most likely to occur in tissues and tumors with elevated levels of ROS and free radicals. Is a protective role of βIII consistent with its observed distribution? As mentioned earlier, βIII is highly concentrated in neurons. The neuronal isozyme of nitric oxide synthase (neuronal NOS [nNOS]) is elevated in the brain *(113)*. NO is produced by neurons, particularly at the synapses *(114–116)*. Although NO has not been shown to react directly with tubulin sulfhydryls, it does react with the microtubule-associated protein (MAPs) tau and it has been proposed that NO could thus play a regulatory role in neuronal differentiation *(117)*. Although there is no reason to imagine that ROS are especially high in neurons, it must be remembered that adult neurons rarely, if ever, reproduce. The turnover time of neuronal tubulin is unknown, but it is probably very long, perhaps in the range of weeks or months. This long turnover time of tubulin would give sufficient time for even a low concentration of ROS to react with tubulin and damage the microtubules. Thus, there is a clear advantage for neurons to form their microtubules from a tubulin isotype less likely to react with ROS, NO, or $ONOO^-$. Incidentally, nNOS is also elevated in muscles, which lack βIII. However, muscles appear to have little need for microtubules so the high NO may not be a problem there.

βIII is elevated in Sertoli cells *(118)*. These cells produce NO and are very rich in the inducible isotype of NOS (inducible NOS [iNOS]) *(119,120)*. The rest of the testis has very little iNOS *(120)*. Sertoli cells also have high levels of the enzyme superoxide dismutase, which they also secrete. This indicates that Sertoli cells operate in an environment rich in free radicals *(121)*. The model would predict, therefore, that Sertoli cells would be rich in βIII. βIII has also been seen in the vestibular organ of the gerbil inner ear. In this organ, which is responsible for balance, the βIII is concentrated in the calyx, a neuronal extension that cups the bottom ends of the hair cells; the dendrites, soma and axons of these particular neurons contain βI and βII in addition to βIII, but the calyx has only βIII *(15)*. It would seem, therefore, that βIII has some particular function in this region. It is probably not coincidental that vestibular neurons as well as the hair cells produce both NO and ROS

(122–125). Small amounts of βIII are present in the colon *(59)* and the nasal epithelium, where βIII is even found in the cilia *(71)*. Although these tissues are not known to produce ROS, it is possible that the colon and the nasal epithelia would be exposed to free radicals in the food we eat and the air we breathe. Interestingly, however, βIII, although present in fetal lung, is absent in adult lung *(126)*. Nevertheless, it would appear that the normal distribution of βIII in adult mammalian tissues is generally consistent with its playing a role in protecting microtubules from oxidation by NO, ROS, or $ONOO^-$.

The presence of βIII in tumors is consistent with this model as well. Many tumors express βIII, including some of nonneuronal origin such as lymphomas *(127–135)*. This has been reviewed by Katsetos et al. *(136,137)*. Tubulin is the target for some of the most successful antitumor drugs such as the taxanes and *Vinca* alkaloids *(138,139)*. Hence, one could argue that cancer cells rely heavily on microtubules. Cancers generally function under oxidative stress, in which the ratio of ROS to antioxidants is abnormally high *(140–144)*. Cancer cells therefore need protection from the same ROS that may have helped to create the cancer in the first place. There is thus a selective advantage for cancer cells to make their microtubules from βIII. One might expect that the more aggressive cancers would have more oxidative stress and hence more ROS and more need for βIII. In fact, it has been observed that tumors of higher malignancy express higher levels of βIII *(134,137,145)*. A study of patients with nonsmall cell lung cancer showed that those whose tumors had elevated βIII responded less well to drugs and had a poorer prognosis *(146)*. When we compared the MCF-7 and BT-549 breast cancer cell lines, it was found that the latter, which has much more βIII than the former, and is resistant to taxol, vinblastine, and cryptophycin 1 *(147)*, also has a much higher level of free radicals (Chaudhuri and Ludueña, unpublished results).

A very interesting finding that may be relevant at this point is that of Carré et al. *(148)*. They found that tubulin occurs in mitochondrial membranes and that the membrane tubulin is enriched in βIII compared with the rest of the cellular tubulin. Mitochondrial membrane tubulin represents about 2% of total cellular tubulin *(148)*. Mitochondria are the cells' major producers of ROS. Perhaps the function of βIII in the mitochondrial membrane is to protect the cell from ROS; conceivably this could be the role of the unusual cys124 of βIII. The ROS could be neutralized by forming a disulfide bridge involving cys124 and either cys127 or cys129. That disulfide could then be reduced by subsequent reaction with the thioredoxin system, a set of proteins that cells use to protect themselves from free radicals. The thioredoxin system has been shown to reduce disulfide bridges in tubulin *(110)*. This is obviously highly speculative, but the possible role of mitochondrial membrane βIII in protecting cells from ROS does parallel the postulated role of βIII in protecting microtubule assembly from ROS.

3.2.3.4. The unusual dynamic behavior of βIII may be highly regulated. If βIII helps a cell cope with oxidative stress, why don't all cells use βIII for their microtubules and not bother with the other isotypes? Does βIII have a countervailing disadvantage? There is evidence that it does. When the αβII, αβIII, and αβIV dimers are allowed to assemble in vitro in the absence of MAPs, αβII, and αβIV begin to assemble immediately, but αβIII only assembles after a long lag-time *(149)*. Whether this is a phenomenon involving nucleation or elongation of microtubules is not clear. In the presence of either tau or MAP2, however, αβIII assembles without a lag-time, exactly as does αβII *(150)*. In another experiment, when βIII was transfected into Chinese hamster ovary (CHO) cells,

microtubule assembly in the cells actually decreased *(151)*. It thus appears that βIII may have an intrinsically lesser ability to polymerize into microtubules. Cells such as neurons, which require large amounts of βIII, may compensate for its poorer polymerization by synthesizing tau, MAP2, or other MAPs. It is interesting that βIII and MAP2 are often synthesized concurrently, not only in neurons *(91,152)* but also in nonneuronal tumors *(90,135)*.

Another unique property of βIII is that microtubules formed of αβIII are considerably more dynamic in vitro than those formed of either αβII or αβIV *(100)*. This property may be very important in development. βIII is expressed in the embryonic nervous system in neurons as well as in cells that later stop expressing it *(136)*. βIII is also expressed in differentiating neuroblastoma cells *(153)* and in regenerating neurons *(154,155)*. It is possible that neurons undergoing rapid growth and differentiation require very dynamic microtubules *(136)*. A small amount of MAP2 is probably expressed at this stage *(156)* and may be sufficient to allow the αβIII dimer to form microtubules. At this stage βIII is not phosphorylated; once the neurons have matured, βIII becomes phosphorylated *(157)*.

3.2.3.5. A Model for βIII Function. All these observations and speculations could be put together into a model for the functional role of βIII, based in part on the ideas of Katsetos et al. *(136)*. In the embryonic nervous system, and perhaps in other cells as well *(158)*, the high dynamicity of microtubules made of αβIII helps the cells to grow and differentiate. At some point, the cells begin to express other isotypes such as βII and βIV that are less dynamic, possibly as a way to regulate the overall dynamic behavior of the microtubules. As the cells differentiate, glia, and other nonneuronal cells stop expressing βIII. In neurons, however, which are faced with problems caused by NO and ROS, βIII expression is retained in order to protect microtubules from oxidation. However, the high dynamicity conveyed by βIII is curtailed, perhaps by increased synthesis of other tubulin isotypes or MAPs, but also by phosphorylation, which is known to promote interaction of βIII with MAP2 *(159)*.

Although this is an attractive model, there are some potential problems with it. First, the high dynamicity of microtubules formed of αβIII was obtained using fully phosphorylated βIII from bovine brain *(100)*. The model assumes that phosphorylation would decrease dynamicity, and therefore predicts that nonphosphorylated βIII would have even higher dynamicity. This prediction, however, has yet to be tested. Second, one could argue that, according to the model, all embryonic tissues should express βIII, as they are all undergoing rapid growth and differentiation. However, this does not seem to be the case *(158)*. It may be that the βIII gene is one of a set of genes that is activated in embryogenesis only in the nervous system and a discrete set of other tissues. The remaining tissues in the embryo may need to find another way to create dynamic microtubules, perhaps using a different tubulin isotype.

3.2.4. βIV

Mammals have two forms of βIV, designated as βIVa and βIVb. The former is expressed only in the brain, whereas the latter is expressed in many tissues including the brain *(62)*. The sequence differences between the two are minor, always involving very conservative amino acid substitutions. Although it is conceivable that there is a functional difference between βIVa and βIVb the fact that birds only have a single βIV

suggests that this is not likely to be the case. At any rate, the monoclonal antibody used to localize and purify βIV does not distinguish between βIVa and βIVb *(150)*. Therefore, in this review the two isotypes will be referred collectively as βIV.

βIV has one very clear-cut function: it occurs in axonemes, the microtubule-based apparatus that powers cilia and flagella. In mammals, βIV has been localized in sperm flagella *(9)*, and in cilia of the tracheal epithelium, brain ependyma, oviduct, efferent duct of the testis, vestibular hair cells, retinal rod cells, olfactory neurons, and esophageal progenitor cells *(15,71,72,80,160)*. In fact, βIV has been found in every mammalian axoneme that has been tested *(72)*. This finding is totally consistent with the prediction of Raff et al. *(73)* who proposed that any β-tubulin that forms part of an axonemal microtubule must contain, very close to the C-terminus, the sequence EGEFXXX (where X is D or E). Of the mammalian β-tubulin isotypes, βIVa, and βIVb are the only ones with this sequence. Therefore, it can be concluded that one function of βIV is to form the axonemal microtubules.

Exactly what role does βIV play in the axoneme? The axoneme is a highly specialized structure, consisting of at least 125 different polypeptides *(161)*. In the middle are two singlet microtubules, known as the central pair. Along the periphery are nine doublet microtubules, known as the outer doublets. Each of these doublets consists of a complete microtubule called the A-tubule and an incomplete microtubule called the B-tubule *(162)*. During motility the motor protein dynein that is connected to the A-tubule of each outer doublet interacts with and slides along the B-tubule of the adjacent outer doublet in a pattern that appears to be regulated by the central pair microtubules *(163,164)*. When one considers the structure of the axoneme, one would imagine that the outer doublet is the specialized microtubule that requires a specific type of tubulin. In contrast, the central pair microtubules seem uncomplicated. In addition, the outer doublet cannot be formed in vitro. Recently, however, it has become clear that the central pair microtubules are special and that they have to rotate around each other to determine which outer doublet pairs slide past one another *(163,164)*. Like the distributor of a car, the rotating central pair microtubules serially make contacts, through some bridging proteins, with specific outer doublets. It should not be surprising if this highly complex microtubule machinery requires a particular tubulin isotype. In fact, there is room in this scenario for more than one isotype. It has been mentioned earlier that many axonemes also contain βI *(72)*.

It is possible that βIV is involved in determining axonemal microtubule structure rather than being directly required for motility. This is based on the observation that two of the cilia types in which βIV occurs are nonmotile: the retinal rod and the kinocilia of the vestibular hair cell *(15,80)*. Whether βIV plays a role in intraflagellar transport is not clear *(165)*.

In *Drosophila*, the β-tubulin isotype, β2, is the only one that contains the EGEFEEE motif and is the only one found in the sperm flagellar axoneme. If β2 loses this motif, or if β2 is replaced by β1, then the outer doublet microtubules are present but not the central pair. Clearly, the EGEFEEE motif is very important. Interestingly, however, if the axoneme motif is inserted into β1, then the outer doublets and central pair are all present, but the distal end of the axoneme is abnormal. This implies that the EGEFEEE motif is not enough to specify a proper axoneme; the other parts of the β2 isotype must also be important *(166)*. Extending this finding to mammals, one could argue that βIV is required for proper formation of both the central pair and outer doublet microtubules.

What is the role of the EGEFEEE sequence? It appears to be the site for polyglycylation, a post-translational modification in which a series of glycines are attached to the γ-carboxyl group of glutamate residues. This modification is very common in axonemal tubulin. The polyglycyl side chain is thought to be necessary for the assembly of the central pair microtubules as well as the B-tubule *(167)*. This modification will be discussed further in Chapter 5. From these observations it is probably safe to hypothesize that βIV is a major constituent of axonemal microtubules because it has a sequence that can be polyglycylated and that this polyglycylation is necessary to form the central pair and outer doublet microtubules.

However, the experiment on *Drosophila* described above implies that other parts of the βIV molecule are necessary for proper formation of the axonemal microtubules. Therein lies a problem for the mammalian sperm cell. The conformation of αβIV is significantly less rigid than that of either αβII or αβIII *(168)*. The high levels of ROS in the testis we have already commented on. Although the precise susceptibility of the αβIV dimer to oxidation has never been tested, it is likely to be higher than that of the other isotypes. The need to protect βIV from oxidation may account for the presence of the protein thioredoxin-like 2 in sperm cells and tracheal cilia. This protein binds well to microtubules and is presumably capable of reducing any disulfide bridges that form in βIV *(169)*.

Another possible function for βIV was observed in cultured rat kidney mesangial cells. The microtubules of these cells contain largely βI and βIV *(170)*. When the cells are extracted, the βI microtubules disappear completely, but the βIV becomes associated with actin filaments *(171)*. Interactions between microtubules and actin filaments are becoming well known *(172)*. The results described here raise the possibility that βIV may be involved in these interactions. It may be that βI and βIV have opposite effects on microtubule–actin crosstalk.

3.2.5. βV

βV is the most intriguing of the β isotypes. It is highly conserved in evolution, suggesting that it may have a specific function. However, not only is that function unknown, even the normal distribution of βV is not known. Using mRNA measurements, Sullivan et al. *(173)* showed that in chickens βV is found in every tissue outside of the brain. Preliminary results with a monoclonal antibody to βV, however, suggest that it is found in mammalian brain but in relatively few other tissues *(174)*. Further work will be necessary to resolve this. Perhaps the only clue to the function of βV is that it has the same distribution of cysteine residues as βIII. In other words, it has cys124 but lacks cys239. If, as was speculated earlier, cys124 allows βIII to react harmlessly with ROS and if the lack of cys239 allows βIII to form microtubules resistant to oxidation, then perhaps the same is true for βV. In fact, βV could conceivably do the job of βIII in tissues that lack that isotype.

3.2.6. βVI

βVI is the least conserved of the β isotypes. In fact, avian and mammalian βVI are so different from each other that it is not clear that they belong to the same isotype class. They have been grouped together because they are clearly associated with the hematopoietic system. In chickens, βVI forms the microtubules of the erythrocyte; in mammals, whose erythrocytes lack microtubules, βVI is found in platelets and in hematopoietic tissues such as bone marrow and spleen *(45,46)*. βVI has a unique arrangement of cysteines.

Table 7
Vertebrate α-Tubulin Isotypes[a]

Designation	*Species*	*C-terminal sequence*	*Distribution*
Class I	Human α1	VDSVEGEEEGEEY	Mostly brain
	Human α3	VDSVEGEEEGEEY	Widespread
	Mouse α1	VDSVEGEGEEEGEEY	
	Mouse α2	VDSVEGEGEEEGEEY	
	Rat α	VDSVEGEGEEEGEEY	
	Chicken α1	VDSVEGEGEEEGEEY	
	Xenopus α1	TDSVEGEGEEEGEEY	
	Torpedo α	VDSVEGEGEEEGEEY	
	Notothenia α	VDSIEGDEEEEGEEY	
	Notothenia α	VDSIEGDGEEEGEEF	
	Salmon α	GDSIEGEGEEEGEEY	
Class II	Human α2	VDSVEAEAEEGEEY	Testis
	Mouse α3/7	VDSVEAEAEEGEEY	
	Rat α3/7	VDSVERKGEEGEEY	
	Trout α	VDSVEGEAEEGEEY	
Class III	Human α4	IDSYEDEDEGEE	Brain, muscle
	Mouse α4 IDSYEDEDEGEE		
	Rat α4	IDSYEDEDEGEE	
	Chicken α5	LDSYEDEEEGEE	
Class IV	Human α6	ADSADGEDEGEEY	Blood
	Mouse α6	ADSAEGDDEGEEY	
	Xenopus	ADSADAEDEGEEY	
	Notothenia α	ADSLGGEDEEGEEY	
	Notothenia α	ADSLGDEEDEEGEEY	
Class V	Human α8	TDSFEEENEGEEF	Heart, skeletal muscle, and testis
	Mouse α8	TDSFEEENEGEEF	
	Chicken α8	TDLFEDENEAGDS	
Class VI	Mouse αTT1	MGSVEAEGEEEDRDTSC CIMFSSSIGNRHPC	Testis
Class VII	*Xenopus*	TESGDGGEDEEDEY	Ovary
Unclassified	*Danio*	ADSTDDCGEDEEEY	

Source: From refs. *8,43,185,305,306,678,401–410*. The classification adopted here is based on that of Lewis and Cowan *(410)*.

[a]Mouse α1 and mouse α2 differ from each other at 1 position. The mouse α3 and α7 genes have different nucleotide sequences but encode identical proteins, referred to as α3/7.

As is true for βIII and βV, βVI has ser239 instead of the assembly-critical cys239. Keeping to this same pattern, chicken βVI has cys124, although mammalian βVI has ser124 *(45)*. Mouse and chicken βVI also have two extra cysteines: at positions 37 and 315 *(45,63)*. One of these may be involved in the disulfide bridge that has been observed in mammalian platelet tubulin *(175)*.

Platelet tubulin has been extensively studied. βVI constitutes about 90% of the total platelet β-tubulin *(176)*. The transcription factor NF-E2 induces βVI synthesis in the megakaryocytes of the bone marrow *(177)*. Platelets are formed by budding off from

megakaryocytes *(178)*. Platelets have a marginal band at the periphery of the cell. The marginal band consists of a single microtubule about 100 μm long, wound around itself 7–12 times *(176,178,179)*. Inhibition of βVI synthesis results in platelets with a marginal band consisting of a single microtubule with only 2–3 coilings; platelets are spherical instead of discoid and, in some experiments, blood coagulation is compromised *(176,179,180)*.

Based on these results one could hypothesize that the peculiar structure of βVI lends itself to forming the marginal band microtubule. This hypothesis appears to be correct. Platelets lacking βVI contain a marginal band formed of the βI and βII isotypes. In the normal platelet, 95% of βVI is in the marginal band, whereas about 45% of βII and 58% of βI are incorporated *(179)*. It would thus seem that βVI is better adapted to forming this unusual microtubule organelle than are the other β isotypes.

3.2.7. βVII

Very little is known about βVII. Its sequence lacks most of the C-terminus and it is expressed in the brain *(181,182)*. Its function, distribution, and properties are completely unknown.

3.3. Mammalian Tubulin Isotypes: the α-Isotypes

There is not much to say about the specific functions, if any, of the α isotypes in mammals. Their tissue distributions seem much less complex, as far as is known, than the distributions of the β isotypes (Table 7). α1 is found mostly in brain but also in a variety of other tissues *(8)*. α2 is similar *(183,184)*. α3/7 is found only in the testis, where it is the major α isotype. α4 is widespread, especially in muscle and heart; α6 is also widespread, but less common than the others. α8 is considerably divergent in sequence, being only 89% identical to the other α (except for the even more divergent αTT1); it is found in heart, testis, and skeletal muscle, and at very low levels in the brain and pancreas *(185)*. The unusual isotype αTT1 is found only in the testis, where it is a minor component of the α population *(42)*.

The α1, α2, α3/7, α4, and α6 isotypes are at least 94% identical in amino acid sequence. In addition, the differences tend to be conservative such as ser/thr or ilu/val. When viewed in conjunction with the fact that the tissue distributions of several of these are quite similar, it is hard to imagine that the differences among these isotypes are functionally significant. However, α8, with its more divergent sequence and its highly restricted distribution, may be an exception. This isotype has a unique sequence at positions 35–45, which is TFDAQASKIND and TFGTQASKIND, respectively, in human and mouse α8. The equivalent sequence in α1, α2, α3/7, α4, and α6 is the completely different QMPSDKTIGGG. This region corresponds to a loop located on the inner microtubule wall that may play a role in contacts between adjacent protofilaments *(185)*. Conceivably, microtubules with α8 could have very different dynamics than those containing the other α isotypes. The fact that the unique features of α8 are highly conserved in evolution suggests that these are functionally significant.

Even though some functional differences among mammalian α isotypes are plausible, none have ever been demonstrated in vitro or in vivo. One approach to this question would be to develop more antibodies specific for α isotypes and then use them for detailed immunohistochemistry as well as to purify tubulin dimers homogeneous for their α-subunit. If the α isotypes exhibit complex cellular distributions, as is the case,

for example, with the β isotypes of the cochlea that would be highly instructive. Similarly, comparison of the behavior in vitro of different α isotypes may reveal functional differences. However, such a comparison has to account for differences that may arise because of different extents of tyrosinolation/detyrosinolation and deglutamylation. In addition, it would be good to know which combinations of specific α and β isotypes occur in different tissues. At the moment, much remains to be done to allow us to understand the significance of the mammalian α isotypes.

3.4. Tubulin Isotypes May Have Functional Roles That Do Not Involve Microtubules

As the mammalian β isotypes have been surveyed, occasional indications of tubulins that may have functional properties of at least potential physiological relevance that do not involve being part of a microtubule have been seen. For example, βII, occurring in the nuclei of various cell types, has been seen as a reticulum rather than as a microtubule; it is in the form of an αβII dimer with apparently normal drug-binding properties *(83)*. Although most of the cells in which this occurs are abnormal, soluble αβII has been shown to compete with heterochromatin protein 1 for binding to the nuclear envelope *(186)*. Similarly, βIII occurs in mitochondrial membranes where it may act to protect the cell from ROS *(148)*. Cells treated to destroy the microtubules show βIV bound to actin. Whether an interaction between actin and soluble αβIV dimers occurs in intact cells is not clear. Nevertheless, the idea of tubulin acting in a nonmicrotubule context is not a new one. A spiral polymer of tubulin constitutes the conoid structure of the protist *T. gondii (187)*. A possibly analogous situation is provided by the enzyme glutathione peroxidase. Usually, the role of this selenium-containing protein is to protect cells from ROS *(188)*. The sperm isozyme of glutathione peroxidase has an additional function, however. After the sperm cell has matured, this isozyme polymerizes to form a sheath around the mitochondrion, losing its enzymatic activity in the process *(189)*. In a sense this is the converse of the situation in microtubules. On the one hand, there has been glutathione peroxidase that normally functions as a monomer, one isoform of which polymerizes, losing its original function. On the other hand, there has been tubulin that normally forms polymers, but that has at least one isoform that can abandon its role in forming that polymer and assume another function. Regardless of the applicability of this particular analogy, the idea that certain tubulin isotypes may have functions that do not involve forming microtubules may be worth pursuing.

An unusual finding that may speak to a possible nonmicrotubule role of βII is its occurrence in the nuclei of a wide variety of cells. This was first discovered in rat kidney mesangial cells *(83)*. In these cells, an antibody to βII strongly stained the nuclei but not the cytoplasm. The staining occurred throughout the nuclei, but was concentrated in the nucleoli. When the mesangial cells enter mitosis, the βII leaves the nuclei and helps to form the mitotic spindle. During telophase, βII enters the reforming nucleus. In contrast, βI and βIV, that constitute the interphase microtubule network, enter the spindle during mitosis, at the end of mitosis returning to the interphase network. These two isotypes never enter the nuclei.

The nuclear βII was in the form, not of a microtubule, but of a reticulum *(83)*. Western blot analysis of the purified nuclei indicated a band reactive with the antibody to βII that comigrated on gels with bovine brain βII. Cells from which the cytosol had been extracted showed α-tubulin in the nuclei as well. Treatment of the cells with fluorescent

colchicine showed accumulation of fluorescence in the nuclei in a pattern indistinguishable from that of βII, suggesting that the nuclear tubulin was in the form of an αβII dimer, capable of binding to colchicine. Disruption of the nuclear βII staining with nocodazole, taxol, and vinblastine, corroborated this interpretation *(190,191)*. Microinjection of fluorescently labeled αβII into the cytosol of rat kidney mesangial cells resulted in accumulation of fluorescence in the nuclei. In contrast, microinjected fluorescent αβIII and αβIV did not enter the nuclei *(170)*. It thus appeared that there was a process that, in these cells, resulted in an αβII dimer entering the nuclei. The fact that micro-injected αβII only entered the nuclei after a cycle of cell division had been completed suggests that nuclear transport may not be involved in the process but rather that the nucleus assembles around the αβII dimer *(170)*.

Other studies revealed that only certain cultured nontransformed cells contained nuclear βII, whereas nuclear βII occurred in almost every cultured cancer cell *(192)*. A survey of about 200 tumors excised from patients showed nuclear βII in 74% of them *(76)*. In general, nuclear βII staining was very variable, depending on the tumor type. In tumors of the prostate, stomach, and colon, nuclear βII was seen in every sample studied. In contrast, only a few hepatic and brain tumors showed nuclear βII. In some excisions, nuclear βII occurred in almost every tumor cell, but sometimes in only a fraction. The intensity of nuclear staining also varied. The pattern of intranuclear staining was variable as well. In some cases, βII was concentrated in the nucleoli; in others it appeared to stain the entire nucleoplasm except the nucleoli. Cytoplasmic staining of βII was also highly variable. Many samples appeared to have βII only in their nuclei and not in the cytoplasm.

The study with human tumors revealed two unusual patterns. First, nuclear βII occurred in tumors of tissues such as the prostate, in which the normal tissue does not express βII. This would suggest that transformation leads cells first to express βII and then to localize it to the nuclei. Second, otherwise normal cells near the tumor would also contain nuclear βII. This was particularly striking in cases of breast cancers that had metastasized to the lymph nodes. Lymphocytes normally do not stain for βII. However, lymphocytes adjacent to the metastatic cancer cells contained nuclear βII. These results suggest that a cancer cell can influence adjacent normal cells to make βII and put it in the nuclei *(76)*. Analysis of a number of normal tissues indicated that most of them did not contain nuclear βII *(76)*. The exceptions were bone marrow, placenta, and pancreatic acinar cells.

What conclusions can be drawn from the story of nuclear βII? It is clearly not a normally widespread phenomenon, being found mostly in cancers and cultured cells. The presence of nuclear βII in cultured cells, tumors, placenta, and bone marrow may indicate an association with proliferation, but this does not explain its presence in the pancreas. A recent finding may cast some light on nuclear βII. Kourmouli et al. *(186)*, working with human endometrial carcinoma cells, examined heterochromatin protein 1, which binds to proteins associated with chromatin such as transcriptional regulators. It also binds to the nuclear envelope. The binding of heterochromatin protein 1 to the nuclear envelope is strongly inhibited by a soluble protein that was found to be a mixture of the α2βII and α6βII dimers. The specific α isotypes involved in this are probably incidental, but it is striking that the only β isotype in these dimers is βII. The authors report that the αβII dimer binds very tightly to the nuclear envelope, thus preventing heterochromatin protein 1 from binding there. These findings raise the possibility that a role of βII may be to control the interaction of chromatin with the nuclear membrane

and perhaps also control the distribution of nuclear membrane fragments during mitosis. Such a possibility may explain the higher concentration of βII observed in the perinuclear region *(81)*. In addition, one could imagine that a relatively minor alteration in certain cell types, especially cancer cells, would result in βII remaining in the nuclei after mitosis is complete. Such alterations may involve a modification of βII or of heterochromatin protein 1 or of the nuclear envelope itself. For example, if heterochromatin protein 1 is altered so as to decrease its affinity for the nuclear envelope, the αβII dimer may stay bound to that membrane at the end of mitosis and may remain in the nucleus during interphase. Of course, the connection of heterochromatin protein 1 and βII may be only a coincidence. However, breaking up the nuclear envelope during prophase and putting it together again during telophase are functions mediated by microtubules *(193–196)*, so a specific connection between the nucleus and one tubulin isotype should not seem too outlandish. On the other hand, many cells appear to lack βII; how do these cells regulate nuclear envelope breakdown and reassembly if βII is important for this process? Are there subtle differences in the processing of the nuclear envelope in these cells? This may be worth examining.

3.5. Not All Isotype Differences are Functionally Significant

The data presented above argue strongly that certain tubulin isotypes have specific functions. This does not necessarily apply to all cases of isotypes, however. Many organisms have isotypes that differ from each other at only a few positions, and only with conservative amino acid substitutions. It is hard to imagine that these small differences are physiologically significant. The key evidence bearing on this point has to do with interchangeability of isotypes. For example, one of the two β isotypes of the fungus *Aspergillus* appears largely during conidiation. However, replacing it with the other one does not alter this process *(197,198)*. Similarly, *Aspergillus* has one α isotype involved in vegetative growth and another in sexual development. Using appropriate manipulations, the expressions of the two α isotypes were reversed. No effect on the viability of *Aspergillus* was observed, provided that particular levels of expression of each isotype were chosen. In fact, it took three copies of the vegetative isotype to replace the sexual isotype without altering the phenotype *(199)*. This experiment has an important implication, namely that when performing genetic manipulation of isotype expression, one must be careful to maintain the same level of total tubulin isotype expression. For example, it may be that the vegetative α isotype of *Aspergillus* is expressed at a lower level than is the sexual α isotype. In that case, replacing the latter with only one copy of the former would mean that the total amount of tubulin expressed would be lower than normal and that could have a deleterious effect. Alternatively, the extra β-tubulin, lacking its α partner, may be toxic to the cell. These factors have to be considered when weighing the results of this type of experiment.

Several early experiments using cultured cells suggested that the vertebrate isotypes were interchangeable. For example, in cultured cells, most of the β isotypes are able to form the mitotic spindle as well as the interphase microtubule network *(118,200–202)*. A similar result was obtained with the α isotypes *(203)*. These experiments, however, do not necessarily prove that the tubulin isotypes are interchangeable. As most cells, no matter what isotypes they express, have both a mitotic spindle and an interphase network, it is not surprising that each isotype could participate in forming these structures. However, cultured cells are less complex than cells *in situ*. The latter may have a specialized need for a particular tubulin isotype that would not arise in a cultured cell.

The above experiments, although very carefully performed, were not set up to address the kinds of subtle and varied possibilities that have been being reviewed for the mammalian β isotypes such as the ability to form axonemes, protect cellular microtubules from oxidation, interact with actin, or form marginal band microtubules.

3.6. Isotype Differences May be Generally Adaptive Without the Isotypes Having Specific Functions

It is possible that certain tubulin isotypes may not have specific functions but that the presence of different isotypes may be adaptive in that they may increase the repertoire of responses to environmental challenges. This is likely to be the case in some plant isotypes. For example, expression of certain β isotypes in *Arabidopsis* decreases in the cold whereas that of another β isotype increases *(204)*. A similar result was obtained in wheat, where lowering the ambient temperature to 4°C increased the expression of one α isotype and decreased that of another *(205)*. Under these conditions, the microtubules become more dynamic. The authors propose that microtubules act as a kind of temperature-sensor and that the cold-induced change in their behavior triggers specific cellular responses to the cold *(205)*. Such a model would not be possible without having different isotypes and yet a specific function cannot be assigned to each isotype.

Multiple isotypes may also play a role in resistance to toxins. For example, there is evidence that having multiple isotypes may make nematodes more resistant to benzimidazoles *(206–210)*. Warm-blooded mammals are more protected from the environment than are plants or nematodes. However, a great deal of evidence indicates that tumors expressing certain isotypes are more resistant to drugs, or that drug treatment may lead to increased expression of particular isotypes (reviewed in refs. *211,212*). Do these results—which will be discussed in more detail later on—speak to the hypothesis of multiple isotypes being generically adaptive in mammals? Certainly, as will be seen, the specific interpretations of these results are highly complex. It is hard to imagine that we evolved for a half-billion years in order to develop mechanisms of resistance to antitumor drugs. On the other hand, one must recall that many of these drugs are, or are derived from, natural products. Thus, it is not inconceivable that the relative amounts of tubulin isotypes may be adjusted in order to help cope with environmental toxins. The fact that several of these toxins are intended to heal, is an unfortunate complication.

Another mechanism by which tubulin isotypes can be generally, rather than specifically, adaptive, is to have them differ in functionally relevant properties such as their dynamic behavior. For example, the yeast *Saccharomyces cerevisiae* has two α-tubulin isotypes (Tub1 and Tub3) *(213)*. Microtubules made of Tub3 are less dynamic in vitro than are the wild-type microtubules. Conversely, microtubules made from Tub1 are more dynamic. The shrinkage rate and the catastrophe frequency for Tub1 are, respectively, four- and threefold more than the corresponding parameters for Tub3, resulting in Tub 1 microtubules having twice the dynamicity of Tub3 microtubules *(214)*. Perhaps, the cell can alter the relative proportions of the two isotypes in order to adapt its microtubule dynamicity to different conditions.

3.7. Altered Expression of Tubulin Isotypes in Drug-Resistant Cells and Tumors

One of the most interesting observations was reported by the Horwitz laboratory, which found that the levels of βI and βII rose 1.9-fold and 21-fold, respectively, in

taxol-resistant murine cell lines *(215)*. Subsequently, Ranganathan et al. *(216,217)* observed that the levels of βIII and βIVa rose four to ninefold and three to fivefold, respectively, in estramustine-resistant DU-145 human prostate cancer cells. Taxol-resistant MCF-7 human breast cancer cells were found to express increased levels of βIII, βIVa and the tyrosinated form of α-tubulin *(218)*. Surveying the many studies done in this area, one of the most frequent results is that tumors expressing increased levels of βIII are more resistant to taxanes and estramustine *(146,212,218–221)*. Almost as frequently observed is that increases in βIV expression also accompany resistance to taxanes and vincristine *(222–224)*. In many fewer cases, taxane resistance involves increased expression of βI *(225)* or βII *(212,215,226)*. Increased βI expression also correlates with resistance to vincristine and E7010 *(222,227)*. Thus, there is ample evidence to suggest that cells alter the synthesis of certain tubulin isotypes in order to survive drug exposure.

How can one make sense of these complex findings? One can speculate that tumor cells elevate the synthesis of the isotype that has the weakest affinity for the drug in question. For example, Derry et al. *(228)* showed that the dynamics of microtubules made from the αβII dimer are significantly more sensitive to inhibition by taxol than are the dynamics of microtubules made from αβIII or αβIV. This is certainly consistent with the majority of the taxane studies, which showed resistance accompanied by increases in βIII or βIVa. These results are also corroborated by the observation that inhibition of the synthesis of βIII by the antisense phosphorothioate oligodeoxynucleotide increases the sensitivity of A549 lung cancer cells to taxol *(229)*. Similarly, transfection of βIII into CHO cells caused a slight increase in taxol resistance *(151)*. On the other hand, overexpression of βIII in human prostate cancer cells failed to affect the sensitivity to taxol *(230)*. Furthermore, studies with human ovarian tumor xenografts failed to detect any significant role of a specific tubulin isotype level on taxol sensitivity *(231)*. These investigators used patient samples (before or after chemotherapy with taxol) to establish a subset of 12 xenografts, and found no correlation between the tubulin isotype expression and the taxol sensitivity. Similarly, overexpression of βIVb in CHO cells did not create resistance to taxol *(232)*. Resistance to *Vinca* alkaloids is reported to be associated with decreased βIII expression *(233)*. This is not consistent with the finding of Khan and Ludueña *(159)* who showed that microtubule assembly of αβIII in the presence of tau was more sensitive to vinblastine inhibition than was assembly of either αβII or αβIV. In short, the hypothesis that tubulin isotypes that are elevated in drug-resistant tumor cells are those isotypes that interact less well with that drug in vitro is consistent with some studies but not others.

What other factors could account for these contradictions? First, there are certain experimental aspects to be considered. If Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) indicates that the mRNA of one isotype increases much more than that of another isotype in response to a drug that does not necessarily mean that the protein levels of these isotypes increase in the same ratio. One isotype may be more sensitive to proteolysis, for example. Similarly, isotype-specific antibodies may detect a many-fold increase in one isotype and only a small percentage increase in another. However, if the latter is much more abundant in the cell than the former, then the small percentage increase in the latter may be much more significant physiologically than the large percentage increase in the former. In such cases, it is important to know the actual isotype levels rather than only the percentage increase or decrease. Also, it is possible that changes in post-translational

modification in the antibody epitope (which is generally the C-terminus and the site of most modifications) may highly alter the detectability of the isotype even if its actual level remains the same. Second, other processes could be supervening that make the change in level of an isotype irrelevant. For example, taxol resistance is sometimes accompanied by mutations of isotypes such as βI *(221)*. A cell with a taxol-resistant βI may actually increase its resistance to taxol by making less of the other isotypes, including βIII. Third, resistance could reflect the assembly properties of the isotypes rather than their drug-binding ability. For example, microtubules containing βIII are less stable in vitro *(100)*. As taxol can increase microtubule assembly, one could argue that increased βIII would cause more resistance to taxol by making less stable microtubules *(233)*. Similarly, as vinblastine inhibits microtubule assembly, one would expect that decreased βIII would increase the overall stability of cellular microtubules and thus increase vinblastine resistance; this is exactly what has been observed *(233)*. Finally, it is possible that cells that more readily mutate to a drug resistant phenotype have higher concentrations of ROS and, hence, that they may have an increased requirement for βIII in order to protect their microtubules from the ROS. However, if, as it has been speculated, βV has the same protective function as does βIII, then, as βV is rarely measured in tumor cells, a scenario could be hypothesized where βV increases and βIII decreases, keeping the total tubulin concentration the same, but only the βIII decrease is detected. In that connection, it is interesting that a preliminary survey of 12 NIH cancer cell lines found that βV was expressed in 11 of them, generally at higher levels than βIII *(234)*.

The mechanism by which the expression of specific tubulin isotypes is altered in drug-resistant cancer cells is still obscure. Overexpression of the oncogenic epidermal growth factor receptor family of kinases has been reported to induce taxol resistance and also increase the expression of βIVa and βIVb *(235)*. Involvement of p53 has been implicated in modulating the expression of tubulin isotypes and drug resistance in human breast cancer cells *(224)*. Extensive analysis with isogenic stable cell lines overexpressing a specific tubulin isotype may shed light on these mechanisms.

3.8. Properties of Purified Mammalian Tubulin Isotypes In Vitro

If the differences among the tubulin isotypes are functionally significant, then it could be expected that the purified isotypes would behave differently from each other in vitro. To address this issue, monoclonal antibodies have been constructed specific for the mammalian βI, βII, βIII, and βIV isotypes *(59,64,150,236–239)*. These have been used to purify the αβII, αβIII, and αβIV dimers from bovine brain by immunoaffinity chromatography. A large number of parameters have been assayed in vitro. The dimers differ from each other in virtually every parameter that has been assayed. Assembly into microtubules is an obvious first parameter to examine. In the presence of either tau or MAP2, αβII and αβIII assemble more rapidly and to a higher extent than does αβIV *(150)*. In the absence of MAPs, but in the presence of 4 *M* glycerol, αβII and αβIV assemble rapidly with no lag time, whereas αβIII assembles only after a considerable lag-time *(149)*. This raises the possibility that αβIII has a harder time nucleating in vitro in the absence of nucleating factors such as γ-tubulin. Microtubules formed from αβIII are considerably more dynamic than those formed from either αβII or αβIV *(100)*. Possibly consistent with these findings is that the intrinsic GTPase activity of tubulin is the highest for αβIII than for either αβII or αβIV *(240)*. However, during microtubule assembly in the absence of MAPs, αβIII hydrolyzes GTP more slowly than do the other two dimers *(9)*. One must be cautious about extrapolating these results to the situation

Table 8
Tubulin Isotypes: Intrinsic GTPase Activity and Interactions With Antitumor Drugs[a]

Ligand	$\alpha\beta_{II}$	$\alpha\beta_{III}$	$\alpha\beta_{IV}$
Intrinsic GTPase			
Induced by colchicine (nmole/h/mL)	4.5	9.6	3
Induced by MTPT[b] (nmole/h/mL)	5.8	11.5	7.3
Interactions with antitumor drugs			
K_d for colchicine (*M*)	4.2	8.3	0.3
$k_{on,app}$ for colchicine binding (*M*/s)	132 ± 5	30 ± 2	236 ± 7
K_d for DAAC[c] (*M*)	0.4	0.7	0.3
k_2 for DAAC (s^{-1})	0.67	0.05	0.59
K_d for MTPT (*M*)	3	6.4	1.8
k_2 for MTPT (s^{-1})	4.22	2.07	5.28
K_d for thiocolchicine THC18 (*M*)	0.5	17	ND[d]
K_d for nocodazole (*M*)	0.52	1.54	0.29
K_d for IKP104 (*M*)	0.01	0.11	1.4–1.8
Suppressivity of dynamics to taxol[e]	3626	765	784
IC_{50} for vinblastine[f] (*M*)	0.6	2.1	0.6
IC_{50} for vinblastine[g] (*M*)	0.5	1.8	2

[a]*Source:* From refs. *159,228,240,260–262,411,412*.
[b]MTPT, 5-(2′,3′,4′-trimethoxyphenyl)-1-methoxytropone.
[c]DAAC, desacetamidocolchicine.
[d]ND, not determined.
[e]This is a parameter that indicates the sensitivity of the shortening rate to taxol *(228)*.
[f]Microtubule assembly was measured in the presence of tau and a series of vinblastine concentrations.
[g]Microtubule assembly, as above, measured in presence of MAP2.

in vivo. Buffer conditions used in vitro may not be physiological and different cell types may have different MAPs that could create major differences in the relative assembly and dynamic properties of the isotypes.

Structural differences among the isotypes are also evident. For example, the mammalian βIII isotype is phosphorylated, whereas the others are not *(241)*. Using differential scanning calorimetry, Schwarz et al. *(242)* found that αβIII is considerably more resistant to decay than is αβII. The half-times for decay at 37°C of colchicine-binding activity for αβII and αβIII were, respectively, 17 h and 50 h *(242)*. Conformation was also probed using a series of sulfhydryl-reactive crosslinkers of the structure: ICH_2–CONH–$(CH2)_x$–NHCO–CH_2I, where *x* (the number of methylene groups) is either 2, 3, 4, 5, 6, 7, or 10 *(268)*. The reagent with $x = 2$ forms two intrachain crosslinks in β-tubulin *(243,244)*. One, designated β*, is between cys239 and cys354 and the other, designated β^s, connects cys12 to either cys201 or cys211 *(102,245)*. When the series of crosslinkers were reacted with the different isotypes, the β* crosslink formed, as expected, in αβII and αβIV, but not in αβIII, which 1acks cys239. However, the β^s crosslink did not form at all in αβIII, even though βIII has the cysteines involved. Also, in αβII, the β^s crosslink formed at high yield with the $x = 2$ crosslinker, and with the crosslinkers where $x = 4$, 5, 6, and 7, but very little with the $x = 3$ and $x = 10$ compounds. In contrast, in αβIV, the β^s crosslink formed well with each crosslinker *(168)*. These results suggest that at least one of the cysteines involved in the β^s crosslink is probably unavailable in αβIII and that it is available in αβII and αβIV, but even more so in the

latter. These results are consistent with αβIII having a more rigid conformation than either αβII or αβIV, but also suggest that the conformation of αβIV is the least rigid of the three dimers.

Not surprisingly, the isotypes also differ in their ligand-binding properties (Table 8). This has been studied in more detail with colchicine and its analogs. Colchicine binds to tubulin in a slow, irreversible, and temperature-dependent manner *(246–252)*. The binding of drug to tubulin results in a promotion of drug fluorescence that has been used to characterize this interaction *(253,254)*. The binding of colchicine is a two-step process in which initial complex formation is followed by a slow conformational change resulting in the formation of a stable complex *(255,256)*. When the association kinetics are studied under pseudo-first-order conditions, the kinetics exhibit a biphasic pattern *(255–257)*. Biphasic kinetics are also observed for the faster-binding analogs of colchicine such as desacetamidocolchicine (DAAC) and the bicyclic analog 5-(2′,3′,4′-trimethoxyphenyl)-1-methoxytropone (MTPT), which binds to tubulin almost instantaneously *(257,258)*.

The origin of the biphasic kinetics in the colchicines–tubulin interaction was not clear until it was demonstrated that immunoaffinity depletion of the tubulin dimers to remove the αβIII dimer eliminated the slow phase, resulting in monophasic kinetics *(259,260)*. Furthermore, addition of αβIII to the αβIII-depleted tubulin restored the biphasic kinetics. Subsequent kinetic studies with the isotypically pure tubulin dimers demonstrated that the isotypes differ significantly in their on-rate constants for binding colchicine. The apparent on-rate constants ($k_{on,app}$) for αβII, αβIII, and αβIV are shown in Table 8. Scatchard analysis revealed that the isotypes also differ in their affinity constants for colchicine and its B-ring analogs *(261,262)*. Analysis of the binding kinetics of colchicine and its analogs indicated that not only does αβIII have the lowest affinity for colchicine, but that the rate (k_2) of the conformational change in tubulin that is part of the drug binding reaction is the slowest for αβIII (Table 8) *(261,262)*. The slow rate of this conformational change may reflect the higher rigidity of αβIII. If this is the case, then this may explain its lessened ability to interact with nocodazole and taxol, although the binding kinetics of these drugs with tubulin isotypes have not been studied in any detail.

The interaction of *Vinca* alkaloids with purified tubulin isotypes is more complicated. One study compared the effects of vinblastine on αβII, αβIII, and αβIV and measured vinblastine's ability to inhibit microtubule assembly and induce spiral aggregate formation *(159)*. The results were clear: microtubule assembly of αβIII was least sensitive to inhibition by vinblastine. Similarly, αβIII was the least susceptible to vinblastine-induced aggregation. Interestingly, although vinblastine induced αβIV to form spiral aggregates, αβIII generally formed amorphous aggregates instead *(159)*. A second study carefully and rigorously examined the effects of three *Vinca* alkaloids (vincristine, vinblastine, and vinorelbine) on self-aggregation of αβII and αβIII. Few significant differences between αβII and αβIII were noted *(239)*. Although the two studies appear to give contradictory results, this is not necessarily the case. No MAPs were present in the latter study, whereas they are present in the former. The study of Lobert et al. *(239)* suggests that the isotypes do not differ in terms of the specific tubulin–tubulin interactions or conformational changes involved in self-aggregation. The study of Khan and Ludueña *(159)* suggests that the isotypes differ either in their interactions with MAPs or else in the ability of vinblastine to interfere with the MAP-induced change in tubulin conformation that permits

assembly. The work of Banerjee et al. *(64)* suggests that αβII and αβIII interact equally well with both MAP2 and tau, so the former model is unlikely. As αβIII has the most rigid conformation of the three dimers, it is not surprising that vinblastine's ability to interfere with the conformational change induced by the MAPs is the weakest in αβIII. Similarly, as a conformational change induced by vinblastine is likely to favor aggregation that change is likely to be least marked in αβIII. This is consistent with the observation that αβIII does not aggregate into spirals; perhaps the conformation of αβIII does not permit it to form spirals. A startling difference in vinblastine-induced aggregation was seen when vinblastine (20 *M*) was added to preparations of erythrocyte tubulin and brain tubulin from chickens *(105)*. The former consists largely of αβVI, whereas the latter is likely to be a mixture of αβI, αβII, αβIII, and αβIV *(64)*. About 42% of the brain tubulin aggregated into spirals whereas 74% of the erythrocyte tubulin formed spirals. Aggregation of the latter was so dramatic that the resulting flocculent precipitate was readily visible to the naked eye *(105)*. Clearly, αβVI has a unique ability to interact with vinblastine. Conceivably, the ability of βVI to form microtubules in which the protofilaments bend so as to form a circular microtubule may translate into a higher ability for the protofilaments to bend to form the vinblastine-induced spiral.

The most consistent finding, one obtained by a wide variety of experimental approaches, is that αβIII has a more rigid conformation than either αβII or αβIV. Could this have any bearing on the differences that have been discussed in vivo? First, a more rigid αβIII would hydrolyze GTP more slowly during microtubule assembly, as has been observed *(9)*. This would increase the growth rate as that depends on the presence of unhydrolyzed GTP at the microtubule end *(263)*. Second, a more rigid dimer is less likely to bind tightly to an adjacent dimer in the microtubule and thus the longitudinal dimer-dimer interactions will be weaker. Hence, the rate of shrinkage might be faster. In short, the increased dynamic behavior of αβIII microtubules may be a function of the rigidity of αβIII.

The basic limitation of the experiments in which purified tubulin isotypes are studied in vitro is that one only gets answers to the questions one asks. Assembly, GTPase, and drug-binding activities are fairly obvious and easy areas to investigate. The fact is, however that the number of proteins or other factors known to interact with tubulin is rising very quickly. To name but a few, in addition to the well-known MAPs, there have been various chaperones *(264,265)*, collapsin-response mediator protein 2 *(266)*, stable-tubulin-only polypeptide *(267)*, the importin/Ran-GTP system *(268)*, XMAP215 *(269)*, Fhit *(270)*, katanin *(271)*, aurora kinase *(272)*, stathmin *(273)*, clathrin-coated vesicles *(274)*, aggregosomes *(275)*, and the proteins of the axoneme, centrosome, and basal body *(276,277)*. In addition to mitosis and the other classical microtubule functions, microtubules are thought to be involved in processes such as determination of neuronal polarity and intramanchette transport *(278,279)*. Katanin, incidentally, has been shown to interact differently with two different β isotypes in *C. elegans (271)*. Recent work suggests that $G_s\alpha$ binds to the β-subunit of tubulin close to the GTP binding site *(280)*. As it has been discussed earlier, both the intrinsic and assembly-mediated GTPase activity of tubulin differ among the isotypes *(9,240)*, it is not unreasonable to expect that the binding and effects of $G_s\alpha$ may be isotype-specific as well. Someday these systems will be constructed and tested in vitro with purified tubulin isotypes. Dramatic differences among the isotypes in such experiments would strongly support the hypothesis that certain functions are mediated by different isotypes.

3.9. Structure–Function Correlations in Tubulin Isotypes

The differences in amino acid sequence among the isotypes of a given organism are generally clustered at the C-terminal ends. The fact that the sequences of the C-termini are usually highly conserved in evolution, even to minor differences, indicates that the C-termini are important. In addition, the C-termini contain the sites of most of the post-translational modifications, including phosphorylation, tyrosinolation/detyrosinolation, deglutamylation, polyglutamylation, and polyglycylation. The C-termini are highly negatively charged. As negative charges repel, the C-termini are likely to be projecting outward from the tubulin dimer and the microtubule. With such a model, it is very easy to imagine that the C-terminus serves as a signal for other proteins that help to determine the function of that isotype. Since a β-tubulin with the sequence EGEFEEE near its C-terminus is likely to form an axoneme *(73)*. Fackenthal et al. *(49)* found that removal of the C-terminus from the axonemal β2 isotype in *Drosophila* did not prevent that isotype from forming the axonemal microtubules, but those axonemes were not functional. Clearly, the signal sequence is necessary for successful function in the case of this isotype. The C-termini of α- and β-tubulin are also the sites where a variety of proteins bind; these include MAP2, tau, calponin, and the motor protein Ncd *(281–283)*. Interestingly, Burns and Surridge *(284)* noticed a correlation between the nature of the aromatic amino acid near the C-terminus of β isotypes and the amino acid at position 217/218. If the former is a tyrosine then the latter two are both threonines, whereas if the former is a phenylalanine, then the latter are other residues. This suggests that the C-terminus may occasionally lie down along the microtubule and interact with the residues at position 217/218. Thus, the "visibility" of the signal sequence may vary depending on circumstances.

The C-terminal sequence is not the whole story, however. Tubulin isotypes differ from each other at other places besides their C-termini. The lack of the assembly-critical cys239 in mammalian βIII is a case in point. Hoyle et al. *(285)* prepared a chimera of *Drosophila* β2 in which positions 1–344 were replaced by the corresponding sequence of β3. The remainder of the β2 contained the C-terminal sequence. β2 is the axonemal and meiotic isotype. If the C-terminal sequence were all that mattered then the chimeric tubulin should function equally well. In reality, the chimeric protein did not form outer doublet microtubules very well and was not able to carry out meiosis successfully. Thus, parts of the protein other than the C-termini must play a role in determining isotype function.

Other evidence supports this hypothesis. For example, a difference has been observed in the conformational rigidity among the αβII, αβIII, and αβIV dimers in the region in which a crosslink can be artificially formed between cys12 and either cys201 or cys211 *(168)*. Modeling studies indicate that this region is the binding pocket for the exchangeable GTP and that GTP binding is influenced by conformational changes in this region *(286)*. The kinetics of hydrolysis of this GTP, which determine the dynamic properties of the microtubule, will certainly be influenced by the conformational rigidity in this area, which in turn depends on the nature of the isotype. Similarly, the lateral and longitudinal bond energies in the microtubule have been estimated and could easily vary among the isotypes *(287)*. Specific amino acid substitutions at positions involved in lateral tubulin/tubulin interactions have been shown to promote cold stability *(288,289)*.

The simplest hypothesis about the structure/function correlations in tubulin isotypes is that the C-terminal sequence serves as a signal to other cellular proteins to determine at which cellular location, or in which population of microtubules, the isotype will perform its function. The rest of the protein is necessary for that function to be performed properly.

4. THE EVOLUTION OF TUBULIN ISOTYPES

4.1. Evolution of the Vertebrate β-Isotypes

Enough β tubulins from vertebrates have been sequenced to enable one to construct a rough family tree. As a first step, it can be asked, which β isotypes do *not* appear in both mammals and birds. Thus, birds have only a single βI and a single βIV. Mammals (mice, humans) have two βIV. Thus, the divergence of βIVa and βIVb must be dated after 310 mya, the date at which the ancestral lines of mammals and birds diverged *(61)* but before the divergence of the rodents and primates at 84 mya *(290)*. Although βIVa and βIVb differ in their tissue distributions—the former occurring in brain only and the latter in all tissues—there is as yet no evidence of a functional difference between them. Humans and rhesus monkeys have two βI but mice have only one *(69)*. Thus, the βI isotype diverged into two species sometime after 84 mya. As with βIVa and βIVb, the functional significance, if any, of the differences between βIa and βIb is as yet unknown.

As a second step, the vertebrate β isotypes should be grouped, based on their sequences, as follows:

1. Group 1: βI and βIV;
2. Group 2: βII;
3. Group 3: βIII and βV;
4. Group 4: βVII;
5. Group 5: βVI.

Groups 1–3 have been identified in the amphibian *Xenopus* (Table 6). Thus, these isotypes were probably present when vertebrates took their first step on land about 360 mya *(291)*. There is no distinction between βI and βIV in *Xenopus*. The separation of βI and βIV probably occurred at the time of the appearance of reptiles over 310 mya *(61)*. In contrast, βVII has been seen only in humans, so it may have appeared very recently. The mammalian and avian βVI are so different from each other that it is possible that each one may have appeared, separately, after 310 mya. βV occurs in birds, mammals, and amphibians, but not in fish. Thus, it probably diverged from βIII at least as early as 360 mya.

Studies of β isotypes in fish are illuminating. There are various β isotypes present in fish that do not have precise equivalents among other vertebrates. In addition to these, however, Groups 1, 2, and 3 can be recognized. Thus, these groups probably diverged from each other at or sometime after the appearance of the chordates about 590 mya *(292)*. A very intriguing experiment by Modig et al. *(293)* may cast light on the early evolution of vertebrate isotypes. The Atlantic cod, *Gadus morhua* has cold-stable microtubules. Transfection of fish βIV into human cells caused the microtubules of these cells to become cold-stable. The same result was observed upon transfection of βII. However, transfection of fish βIII did not confer cold stability. It is logical to assume that if βII and βIV are major structural components of fish microtubules, then they must be able to provide cold stability. In contrast, fish βIII is incapable of performing this function. As *Gadus* lives its entire life cycle at cold temperatures, it is unlikely that it could have microtubules made entirely of βIII, as such microtubules would be cold-labile. It is conceivable, of course, that certain MAPs could make βIII-microtubules cold-stable. This is unlikely, however, as microtubule cold-stability in Antarctic fish has been shown to reside in tubulin and not in MAPs *(294)*.

Thus βIII cannot be the major component of any microtubule population in fish and the invitation is given to speculate upon its function. In mammals, the earlier hypothesizing

suggested that βIII had two major functions: (1) to form highly dynamic microtubules that may be particularly important in development, especially in the nervous system; and (2) to make microtubules resistant to ROS. Both of these functions could reasonably occur in fish. Vertebrate evolution has been suggested to be an example of neoteny, in which a larva attains sexual maturity without metamorphosing into an adult *(295)*. The ancestors of the vertebrates may have had clearly differentiated larval and adult stages, the former motile, and the latter sessile. At one point in evolution, the larva acquired sexual maturity and the adult stage disappeared. Thus, vertebrates were able to grow in size and retain motility. Tunicates, which are nonvertebrate chordates with very simple nervous systems *(296)*, have neither βII nor βIII. It is possible that βIII appeared at the time when the vertebrates diverged from the other chordates and that its high dynamicity made it useful in the rapid growth of the complex nervous system of vertebrates *(297)*. In this connection, it is worth recalling that βIII is common and widespread in embryos, in which growth and development take place very rapidly *(136,158)*. βIII is unlikely ever to have been the sole component of a microtubule, but it could confer dynamicity to microtubules in which it occurred. Microtubules made of mixtures of αβIII and αβII dimers are significantly more dynamic than those made of αβII alone, provided that αβIII predominates *(100)*. It is perhaps not a coincidence that chordates appeared soon after the concentration of O_2 in the Earth's atmosphere reached 10% of present levels, the level required to form collagen and hence cartilage and bone *(298)*. The higher O_2 level would have led to increased production of ROS. As vertebrates developed an advanced nervous system, βIII may have acquired the additional function of protecting the long-lived neuronal microtubules from ROS. If these arguments were correct, one would predict that cephalopod mollusks that are as ancient as the vertebrates and that are long-lived and have a complex nervous system *(299)*, would also have a tubulin isotype capable of protecting the neuronal microtubules from ROS.

The development of the nervous system would also have entailed the appearance of βII, which presumably has a major, but as yet unknown, function in the nervous system. If microtubules play a role in reorganizing the nuclear envelope, and if this is an ancient function, βII may have retained this function, and the other isotypes may have lost it.

About 360 mya, the vertebrates emerged onto the land *(300)*, thereby exposing themselves directly to the higher levels of O_2 present in the atmosphere as well as to the strong solar ultraviolet radiation that is capable of creating free radicals. There may have been a premium on protection of microtubules from ROS, not only in the brain, but in other tissues as well. If βV shares this function with βIII, as has been hypothesized, it is possible that βV appeared about this time to protect the microtubules of other tissues from ROS, whereas βIII performed that same function in neurons.

Full sequencing and analysis of reptile, amphibian, and fish genomes as well as those of the nonvertebrate chordates may flesh out, corroborate, or disprove some of these speculations. Further experiments on the evolution of nitric oxide synthase as well as careful studies of the tissue distribution of nitric oxide synthase and tubulin isotypes in fish would be very useful as well.

4.2. Evolution of the Vertebrate α-Isotypes

The evolution of the α isotypes in vertebrates is not as well understood as that of the β isotypes. It is clear that the class V α (called α8) are present in mammals and birds, but not, so far as it can be told, in amphibians or fish *(185)*. Therefore, α8 probably

appeared between 360 mya and 310 mya. The same argument could be made for class III. However, it appears that classes I, II, and IV were present in fish probably after the chordates appeared around 590 mya *(292)*. *Xenopus* ovarian α and mouse testis αTT1 are too unique to draw conclusions regarding their evolution.

4.3. Evolution of Tubulin Isotypes in the Other Eukaryotes

The knowledge of tubulin isotype evolution in other eukaryotes is quite limited. There are only a few phyla where multiple tubulin isotypes have been sequenced in more than one organism. Nevertheless, there are a few generalizations that are probably safe to make. With one possible exception, which will be discussed shortly, it is clear that, although all α-tubulins resemble each other and the β-tubulins do likewise, there is no specific resemblance between one particular isotype in one phylum and another particular isotype in another phylum. In other words, there is no close structural resemblance between, say, βIII in vertebrates and any β isotype in any other phylum. In brief, families of isotypes are phylum-specific. This has been seen to be true for the α- and β-tubulins in vertebrates. There are discernible families of α and β isotypes in arthropods, nematodes, and angiosperms *(8)*.

The possible exception to this pattern is βIV, the isotype with the signal sequence EGEFEEE, which is required for a β-tubulin to form part of an axoneme *(73)*. This sequence, or one very similar, occurs in at least one isotype in virtually every eukaryotic organism except fungi (which lack axonemes, basal bodies, and centrioles). In a sense, therefore, a tubulin containing this sequence has to be thought of as ancestral to all β-tubulins. It is highly unlikely that the signal sequence would spontaneously arise *de novo* three separate times, during the evolution of animals, plants, and protists. Therefore, a β-tubulin containing this sequence must have been present in the ancestral eukaryote. Fungi, presumably, would have lost this β-tubulin when they lost the complex microtubule apparatuses in which this tubulin is required. That said, however, beyond the signal sequence there is no overall specific quantifiably demonstrable similarity between, say, vertebrate βIV and the corresponding β2 isotype in *Drosophila*. Thus, if assigning to βIV the additional function of being involved in actin–microtubule crosstalk is correct, this function may have arisen secondarily in βIV. If a β-isotype in *Drosophila* also has this function, then that isotype need not be β2.

Various fish (*Notothenia* and *Danio*) have at least one α or β isotype that have no specific equivalent in amphibians, birds, or mammals. The specific functions of these isotypes are unknown. Conceivably, these may represent the survivors of a large pool of tubulin isotypes that arose when the vertebrates appeared. Speculating further, an intriguing correlation could be postulated. Most of today's animal phyla—at least those where fossil evidence is available—arose during the so-called Cambrian explosion, about 530 mya, when the ancestors of today's phyla shared their world with animals with unusual body plans who left no descendants *(301)*. Whatever geological, climatic, or ecological factors promoted the appearance of multiple body plans could also have impelled the diversification of tubulin isotypes. If the multiple isotypes had different functions, then in view of tubulin's important role in development, it is not difficult to imagine that different combinations of isotypes correlated with the appearance of specific phyla. If it is assumed that the earliest eukaryote had a single α and a single β isotype, then this tubulin would perhaps have been involved in different functions: not only mitosis and axonemal motility but perhaps nuclear envelope organization and actin–microtubule

crosstalk as well. The appearance of multiple isotypes meant that these functions could have been distributed among different isotypes. Subsequent evolution of each phylum would involve essentially random selection from this pool of isotypes of particular ones performing whatever functions were adaptive for organisms in that phylum. For example, an early animal having several isotypes with the appropriate signal sequence for axonemal motility, could be imagined. In vertebrates, the ancestral βIV could have been randomly selected for subsequent evolution and the others lost; in arthropods the ancestral β2 would have been similarly selected. In such fashion, phyla would arise with unique families of isotypes, and there would be no specific similarity—other than the signal sequence—among the axonemal isotypes of the various phyla.

Two fish β isotypes may fit this hypothesis. *Gadus* has a β isotype (classified as βIII) that has the same cysteines as does βIII (i.e., it lacks cys239 and has cys124). Nevertheless, the C-terminus of the isotype lacks the basic residues seen in mammalian βIII. Conceivably, this isotype could have the putative antioxidizing property of mammalian βIII but lack the dynamic properties of βIII. In contrast, *Notothenia* has a β isotype (unclassified) that has the C-terminus with basic residues similar to those of βIII or βVI, but does not have the same cysteines as βIII. Perhaps, this isotype exhibits the dynamic behavior of βIII but lacks any antioxidant activity.

In pursuing the evolution of the isotypes of α- and β-tubulin a trail has been followed that fades out sometime before the beginning of the Paleozoic. Further insights may be provided when the story of α and β is compared with that of the other members of the tubulin superfamily, as will be discussed in Chapter 7.

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APPENDIX

Nomenclature of Avian and Mammalian β-Tubulin Isotypes

Class	*Human*	*Mouse*	*Rat*	*Chicken*
Ia	HM40	Mβ5	rbt. 5	cβ7
II	Hβ9	Mβ2	rbt.1	cβ1/cβ2
III	Hβ4	Mβ6	rbt. 3	cβ4
IVa	H5β	Mβ4	rbt. 2	–
IVb	Hβ2	Mβ3	–	cβ3
V	–	–	–	cβ5
VI	Hβ1	Mβ1	–	cβ6
VII	Hβ4Q	–	–	–

Adapted from ref. *410*.