The ring saga: looking back at the discovery of γ-tubulin and γ-tubulin ring complexes

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ABSTRACT **For many years, two central, unanswered questions in cytoskeleton research were how microtubule assembly is nucleated and microtubule polarity established. The discoveries of γ-tubulin and γ-tubulin ring complexes were key advances that allowed these questions to be substantially answered. The discovery of γ-tubulin was the product of a genetic screen in *Aspergillus nidulans* for genes important for microtubule function. γ-Tubulin is a member of the tubulin superfamily of proteins, closely related to α- and β-tubulin but distinct from both. It is ubiquitous in eukaryotes, and in many organisms there are small families of γ-tubulin genes. γ-Tubulin and associated proteins form ring-like complexes that localize to microtubule-organizing centers (MTOCs) and play an important role in the nucleation of microtubule assembly from MTOCs and the establishment of microtubule polarity.**

Monitoring Editor
Keith Kozminski
University of Virginia

Received: Oct 28, 2022
Accepted: Nov 4, 2022

INTRODUCTION

I am honored and grateful to be invited to write this *Retrospective*. Yixian Zheng was previously invited to write a retrospective on γ-tubulin complexes, and she generously asked Vitoria Paolillo and me to participate (Oakley *et al.*, 2015). The document we produced was really a perspective on the field at that time rather than a retrospective. The discovery and early characterization of γ-tubulin was covered in two sentences. This *Retrospective* is a discussion of the discovery of γ-tubulin and the early characterization leading up to the realization that γ-tubulin complexes play a key role in microtubule nucleation. It will fill the large gap in the previous document, and, happily, it will give me a chance to acknowledge many people who made important contributions.

THE DISCOVERY OF γ-TUBULIN

The discovery of γ-tubulin grew out of work that I started in the laboratory of Ron Morris at Rutgers Medical School. Ron was a pioneer in the use of genetics to study mitosis and the cytoskeleton, and his main experimental organism was the filamentous fungus *Aspergillus nidulans*. I was an electron microscopist with an interest in mitosis first developed as an undergraduate in Bruce Nicklas' lab at Duke. Although many cell biologists at the time were skeptical that genetics would be useful in studying mitosis and the cytoskeleton, the Morris lab made a series of advances, identifying tubulin genes and using mutants as tools to answer cell biological questions (Sheir-Neiss *et al.*, 1978; Morris *et al.*, 1979; Oakley and Morris, 1980, 1981). The Morris lab and alumni continued, of course, to make important contributions (some are summarized in Enos and Morris, 1992).

A purchasing freeze was implemented the week I arrived at Ohio State to start my own lab, due to a state budget shortfall. The lack of lab equipment constrained the types of work we were able to carry out, but, fortunately, incubators had been purchased before the freeze and several good colleagues, John Reeve, Tom Byers, Neil Baker, Don Dean, and others, lent us microscopes and other small pieces of equipment. This allowed us to carry out a mutant screen for proteins important to microtubule function.

At that time, we knew very few of the molecules important for mitosis. Simply identifying molecules that were demonstrably important for mitosis and/or microtubule function was important. The screen we developed was based on findings by John Jarvik and David Botstein (Jarvik and Botstein, 1975). Working with phage P22, Jarvik and Botstein had shown that missense mutations were often suppressed by new mutations in genes whose products interact physically with the original protein. Applying this principal to microtubules, if one had conditionally growth inhibited α- or β-tubulin mutations, one could, in principle, isolate mutations in genes important for microtubule function as suppressors of these tubulin mutations.

DOI:10.1091/mbc.E22-07-0290
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Abbreviation used: MTOC, microtubule-organizing center.

The Morris lab had already employed this strategy in a limited way (Morris *et al.*, 1979). J. M. van Tuyl, a Dutch geneticist/plant pathologist, had previously mapped many *A. nidulans* mutations that conferred resistance to the antimicrotubule fungicide benomyl to a locus he named *benA*. Geraldine Sheir-Neiss in the Morris lab reasoned that *benA* might be a tubulin gene, and with the help of an excellent technician, Margaret Lai, she was able to demonstrate that the *benA* locus is a structural gene for β-tubulin (Sheir-Neiss *et al.*, 1978). This was the first identification of a β-tubulin gene. Among the van Tuyl *benA* mutants, three conferred weak heat sensitivity. Morris *et al.* (1979) isolated non–heat-sensitive revertants of these mutants and demonstrated that reversion was due to mutations in a structural gene for α-tubulin, which they named *tubA*. This was the first identification of an α-tubulin gene.

In the Morris lab I isolated a large number of benomyl-resistant mutants hoping to find a *benA* allele heat sensitive enough to analyze by electron microscopy. This proved successful, and one mutant, which I designated *benA33*, was particularly interesting (Oakley and Morris, 1981). *benA33* blocked mitosis at high temperatures but nuclei blocked in mitosis had morphologically normal spindles. The heat sensitivity of *benA33* was suppressed by antimicrotubule agents and by an α-tubulin mutation that destabilized microtubules. Our relatively straightforward explanation was that *benA33* hyperstabilized microtubules, somewhat similar to paclitaxel, which was just being characterized at that point. These results supported models that postulated that microtubule disassembly either powered chromosome movement or was rate limiting for chromosome movement (Inoué and Sato, 1967; Dietz, 1972; Forer, 1974; Margolis *et al.*, 1978). Additional evidence that *benA33* hyperstabilizes microtubules was obtained subsequently (Gambino *et al.*, 1984). Beyond the value of the mutant for understanding mitosis, the tight blockage of growth at high temperatures allowed us to isolate revertants in large numbers.

At Ohio State, we mutagenized a strain carrying *benA33* and isolated more than 2600 non–heat-sensitive revertants. Genetic analysis revealed useful α- and β-tubulin mutations among the revertants (Oakley *et al.*, 1985, 1987a; Weil *et al.*, 1986), but the most interesting mutations were three suppressors of *benA33* that were not in α- or β-tubulin genes. These proved somewhat difficult to work with genetically, as they would neither cross with each other nor form stable diploids with each other. We were able to map all of them, however, to between 0.221 and 0.325 cM centromere proximal to the *riboB* (riboflavin requirement) locus on linkage group VIII. The closeness of the mutations to each other suggested that they were allelic, and we named them *mipA1-3* (*mip* for microtubule-interacting protein).

The three *mipA* alleles suppressed the inhibition of mitosis and nuclear movement caused by *benA33*, and they displayed a pattern of allele-specific interactions with each of the conditionally growth inhibited *benA* alleles that had been isolated. The pattern of interactions suggested to us that the product of *mipA* had some specific and important, but as yet undefined, role in microtubule function. A problem was that in a wild-type (wt) *benA* background all three of the *mipA* alleles were silent, so cloning them by complementation was not possible. The proximity to *riboB* suggested, however, that if we could clone *riboB* it might be possible to chromosome walk from *riboB* to *mipA*.

Fortunately, in parallel with our screening and analyses, we and others had been improving *A. nidulans* molecular genetics. I was lucky enough for my first National Institutes of Health grant application to go to a study section that was willing to take a chance on an electron microscopist proposing genetic and molecular genetic approaches with a filamentous fungus that, at that time, had yet to be transformed. *A. nidulans* was soon transformed (Ballance *et al.*, 1983; Yelton *et al.*, 1984), but the transformation frequencies were very low. I hired two very talented people, Janet Rinehart and Brenda Mitchell, who had just graduated from Ohio State, and, working together, we were able to boost transformation frequencies about 100×. We then created a plasmid *A. nidulans* genomic library and found that we could complement a mutation in the *pyrG* gene (pyrimidine requirement) with the library. Although no shuttle vector would be available in *A. nidulans* for years, we were able to recover the *A. nidulans pyrG* gene from a transformant by partially digesting genomic DNA of the transformant, ligating at a low DNA concentration to favor circularization over oligomerization, and transforming *Escherichia coli* with the ligation mixture, selecting for an antibiotic resistance gene on the plasmid in which the library was constructed. From the *E. coli* transformants we were able to isolate plasmids carrying the *pyrG* gene (Oakley *et al.*, 1987b). We then cloned the *riboB* gene using the same approach (Oakley *et al.*, 1987c).

To clone *mipA* by chromosome walking, we needed a genomic library with large inserts, and we needed an assay to allow us to identify inserts that contained the gene. Steve Osmani from the Morris lab provided us with a Charon 4 genomic library and shared information on cotransformations that suggested to us that we might use a cotransformation approach for identifying fragments carrying the *mipA* gene. We created a strain carrying *benA33*, *mipA1*, and *pyrG89*. In combination, *benA33* and *mipA1* confer weak, but scorable, cold sensitivity. If we cotransformed this strain with the *pyrG* gene and a DNA fragment that contained the wt *mipA* allele, a fraction of the *pyrG*$^+$ transformants should be cotransformed with the wt *mipA* allele, and these cotransformants should no longer be cold sensitive. This strategy was sufficiently risky that, rather than compromise a graduate student's or postdoc's career, I took the project on myself. I was fortunate enough to be aided by two terrific technicians, my wife Liz and Patti Kretz (neither of whom had previously done any molecular biology). Although it required a great deal of effort, the approach worked, eventually yielding two Charon 4 inserts that complemented *mipA1*. Examining overlaps of the two inserts revealed that the 4.8 kb *Eco*RI fragment contained the *mipA* gene. With the aid of Mark Mooney, a Kings College, London, undergraduate who was working in the lab over the summer, we narrowed the *mipA* gene down to a 2.8 kb *Hin*dIII-*Sac*I fragment. The fragment complemented *mipA1 in trans*, so we were confident that it contained the entire *mipA* gene.

Sequencing in that era was not trivial, but we successfully sequenced the fragment. The *mipA* gene is riven by seven introns, however, so it was very difficult to figure out the coding sequence of the gene, or even where the gene was on the fragment, without a cDNA sequence. We cloned cDNAs that hybridized to the fragment from a library provided by Steve Osmani, and they revealed that the fragment contained one complete gene and part of another. The sequence reads of the full-length *mipA* cDNA were made by Liz and Kathy Jung, a postdoc, while I was teaching a genetics class. Kathy had been sequencing mutant β-tubulin alleles, and she was able to translate the sequence quickly using a computer program written by Jim Brown, a graduate student in John Reeve's lab, and compare it to the 20-odd α- and β-tubulin sequences available at that time. When I returned from my class, everyone in the lab knew that we had found something that was remarkable and unexpected. It was clearly a member of the tubulin superfamily of proteins, but it was distinct from α- and β-tubulins.

We wrote up our finding and sent it to *Nature*, with Liz as first author because she had done so much of the work. It was promptly

rejected without being sent to review. In retrospect, the problem was that I had written the manuscript too cautiously and the editors were not understanding that we had discovered a new tubulin rather than just a new isotype of α- or β-tubulin. I wrote back, explaining the significance more carefully and suggesting that there were a number of excellent cytoskeleton researchers in London who could look at it quickly and tell them whether it was worth sending out for review. We eventually received a note that it had been sent out for review, and a month later we received the reviews. The reviews were quite positive, but we were criticized for being "too cautious" and "too modest about the significance of the finding." We were asked to revise the text, shorten it, and add Northern blot data that we had referred to as "not shown," all in two weeks. Our Northern blot data were not pleasing esthetically so we decided to redo the blot, which required us to make new RNA so there was a race to revise the text, produce a clean Northern blot, and revise the figures. In retrospect the comments of the reviewers were very helpful and gave us license to make some assertions that we had wanted to make in the first place but were too cautious to include in the original submission. The result was a much improved paper.

The existence of a new tubulin raised obvious questions such as how widespread it is and what its function is. We worked on the two questions in tandem, but I will deal with them separately.

UBIQUITY OF γ-TUBULIN

In our original paper, comparisons of the sequences of γ-tubulin with α- and β-tubulin sequences led us to conclude (from Oakley and Oakley, 1989), "these data, in sum, are inconsistent with a recent origin of γ-tubulin and the simplest hypothesis consistent with these data is that γ-tubulin diverged from a tubulin (α, β, or ancestral) at about the time of the α/β divergence, near the time of the first eukaryotes. Since there is no obvious reason that γ-tubulin should have been retained only in *A. nidulans*, it is probable that it (and perhaps other, undiscovered tubulins), will be present in many, perhaps all, eukaryotic organisms." With respect to the ubiquity of γ-tubulin, Yixian Zheng, who was a graduate student in my lab, was happy to take the lead. As in much of the early γ-tubulin work, progress was accelerated by exchange of information and reagents with other labs. A colleague at Ohio State, Gene Leys, provided us with a *Drosophila melanogaster* ovary cDNA library constructed by David Joseph and others at the University of North Carolina. Yixian probed it at low stringency with an *A. nidulans* γ-tubulin cDNA and obtained very faint hybridization. She was able to quickly (by the standards of the time) clone and sequence four cDNA inserts. They were all copies of a γ-tubulin cDNA that encoded a protein with 66.7% amino acid identity with *A. nidulans* γ-tubulin. Yixian used the *D. melanogaster* cDNA to probe a human (HeLa) cDNA library provided by Harish Joshi and Don Cleveland and was able to clone and sequence a human γ-tubulin cDNA. It was now clear that γ-tubulin was present in evolutionarily divergent organisms and, indeed, from the many subsequent genome projects it appears to be present in all eukaryotes (Findeisen *et al*., 2014).

FUNCTION OF γ-TUBULIN

With respect to the function(s) of γ-tubulin, we took two paths, both using *A. nidulans* initially. We wanted to localize γ-tubulin by immunofluorescence, and we wanted to determine what effects removal of γ-tubulin would have on the cell. Kathy Jung took the lead on making γ-tubulin antibodies, helped by Yisang Yoon, a graduate student. Because purifying γ-tubulin from *A. nidulans* didn't seem feasible, we expressed *A. nidulans* γ-tubulin in *E. coli*, purified it, and injected it into three rabbits. Most rabbits have antibodies against fungi, and ours were no exception, giving multiple bands in Western blots and interesting patterns by immunofluorescence. We worked out procedures to affinity-purify γ-tubulin–specific antibodies and found that γ-tubulin localized mainly to the spindle-pole body (SPB), with a little in the cytoplasm. The SPB was a particularly intriguing location because it is the fungal analogue of the centrosome. Both are microtubule-organizing centers (MTOCs) that have the ability to nucleate microtubule assembly and establish microtubule polarity. The components of MTOCs that nucleate microtubule assembly were, at the time, the subject of a great deal of speculation and debate.

To determine the effects of the removal of γ-tubulin, we disrupted the *mipA* gene, and it proved to be essential for viability. Steve Osmani and Ron Morris had developed the heterokaryon rescue technique for *A. nidulans* that allows one to keep nuclei carrying a recessive lethal mutation alive in a heterokaryon and determine the phenotype of the lethal mutation in germinating spores produced by the heterokaryon. We applied this technique to the *mipA* gene and found that the absence of γ-tubulin resulted in strong inhibition of nuclear division, the elimination of nearly all mitotic spindles, and a great reduction in the number and extent of cytoplasmic microtubules (Oakley *et al*., 1990) (additional data in Martin *et al*., 1997). These data, coupled with our genetic data and the sequences of α-, β-, and γ-tubulin, suggested to us that complexes of γ-tubulin at the SPB might interact with the tubulin dimer, thereby nucleating assembly. Furthermore, if γ-tubulin bound specifically to α- or β-tubulin, but not both, nucleation by γ-tubulin would establish the polarity of microtubules (Oakley *et al*., 1990). This has, of course, turned out to be correct, but we speculated, on the basis of our genetic data, that γ-tubulin might interact physically with β-tubulin, and, of course, it binds to α-tubulin in reality.

We obviously wanted to determine whether metazoan γ-tubulin was located at centrosomes, but unfortunately, in Western blots, our antisera against *A. nidulans* γ-tubulin recognized bands in addition to γ-tubulin. To circumvent this problem, Kathy Jung expressed a human γ-tubulin cDNA and used it to affinity-purify antibodies from our rabbit sera. These were specific and stained the centrosomes of HeLa cells, *D. melanogaster* S2 cells, and mouse fibroblast cells.

Other labs were now beginning to make significant findings. Tetsuya Horio and others in Mitsuhiro Yanagida's lab showed, in a collaboration with my lab, that fission yeast γ-tubulin is essential for mitosis and localizes not only to SPBs but also to cytoplasmic MTOCs (Horio *et al*., 1991). Tim Stearns and Louise Evans in Marc Kirschner's lab showed that γ-tubulin is associated with pericentriolar material and remains at the centrosome when microtubules are depolymerized (Stearns *et al*., 1991). Harish Joshi and others in Don Cleveland's lab showed that a polyclonal antibody against γ-tubulin could block microtubule regrowth from the centrosome after microtubule depolymerization with nocodazole and cold and that injection of these antibodies immediately before or during mitosis could prevent the formation of a functional spindle (Joshi *et al*., 1992). Marie-Anne Félix and others showed that, in *Xenopus laevis* extracts, γ-tubulin is recruited to forming centrosomes and immunodepletion of γ-tubulin from the extracts inhibited the assembly of microtubules from the centrosomes (Félix *et al*., 1994). Evidence was accumulating that γ-tubulin is a key component of MTOCs that plays a crucial role in microtubule nucleation.

CONSERVATION OF γ-TUBULIN FUNCTION

The conservation of γ-tubulin amino acid sequences over large phylogenetic distances suggested that the functions of γ-tubulin were conserved. Tetsuya Horio, who had now joined my lab, was able to

create a strain of *Schizosaccharomyces pombe* in which the endogenous *S. pombe* gene was removed and the only γ-tubulin gene present was a human γ-tubulin cDNA under control of an *S. pombe* promoter (Horio and Oakley, 1994). The strain was surprisingly healthy, growing only slightly slower than a wt control. Immunofluorescence microscopy revealed that it had normal mitotic spindles and cytoplasmic microtubule arrays and that, like *S. pombe* γ-tubulin, human γ-tubulin localized to SPBs. We were later able to show that even a plant γ-tubulin gene functioned well enough in *S. pombe* to support viability and growth (Horio and Oakley, 2003) in spite of the fact that higher plants do not have morphologically identifiable MTOCs. Curiously, however, the γ-tubulins of two common model organisms, *Saccharomyces cerevisiae* and *Caenorhabditis elegans*, have diverged enough that are not functionally interchangeable with most other γ-tubulins.

γ-TUBULIN GENE FAMILIES

There are families of α- and β-tubulin genes in most eukaryotes, and we were interested in determining whether that is the case for γ-tubulin as well. Our efforts turned up no evidence for a second γ-tubulin gene in *A. nidulans*, but, using PCR with degenerate primers derived from conserved regions of γ-tubulin, we were able to identify a second γ-tubulin gene in *D. melanogaster* (Wilson *et al.*, 1997) that we were able to clone, sequence, and map. Trish Wilson from Minx Fuller's and Gary Borisy's labs was able to carry out a very thorough examination of the expression patterns of the two γ-tubulin genes (Wilson *et al.*, 1997). They are differentially expressed with fairly complex expression patterns that are worthy of greater discussion than space permits here.

Dawnne Wise in my lab, working with Ralf Krahe at Ohio State, was able to characterize the human γ-tubulin gene family. Dr. Helmut Schraudolf provided us a human genomic γ-tubulin DNA fragment, but its sequence did not match the known human γ-tubulin, TUBG1. We found an expressed sequence tag at the National Center for Biotechnology Information that corresponded to the new γ-tubulin and sequenced the corresponding cDNA. The predicted gene product had 97.3% identity with the TUBG1 γ-tubulin, and we designated it TUBG2. The two genes mapped very close to each other in the q21 region of chromosome 17 (Wise *et al.*, 2000).

DISCOVERY OF THE γ-TUBULIN RING COMPLEX

Yixian Zheng completed her PhD in my lab and joined the labs of Bruce Alberts and Tim Mitchison. She was able to purify γ-tubulin complexes from *Xenopus* oocyte extracts and show that they can nucleate microtubule assembly (Zheng *et al.*, 1995). In the electron microscope these complexes were ring shaped (or broken ring shaped), and so they were called γ-tubulin ring complexes or γ-TuRCs. I was surprised that these experiments worked because if γ-tubulin complexes nucleated microtubule assembly, and there were many γ-tubulin complexes in *Xenopus* oocytes, then microtubule assembly would occur at many random sites. In fact, γ-TuRCs are microtubule nucleators, but not very good ones outside of the context of MTOCs.

Michelle Moritz and coauthors were able to demonstrate that ring-shaped complexes containing γ-tubulin are present in the pericentriolar material of *D. melanogaster* centrosomes, and Jackie Vogel and colleagues showed that similar rings were present in *Spisula* centrosomes. Finally, Moritz *et al*. (1998) and Schnackenberg *et al*. (1998) demonstrated that it is possible to strip away the ability of isolated centrosomes to nucleate microtubule assembly with salt and γ-TuRCs are required for the restoration of the ability to nucleate microtubule assembly. A great deal of evidence of various kinds with a variety of organisms converged to confirm that γ-tubulin complexes nucleate microtubule assembly. Now of course the components of the γ-TuRC have been identified and the structure of the γ-TuRC has been almost completely determined at atomic resolution (Zupa *et al*., 2020).

ACKNOWLEDGMENTS AND CONCLUSIONS

I end this chronicle with a note of thanks to all members of my lab and collaborators, including those I have mentioned as well as others. I particularly thank Liz Oakley for being indispensable for every part of this journey and for correcting my memory on parts of this document. I will indulge myself in pointing out some of the obvious things that can be learned from this work. One is that cooperation speeds scientific progress. Another is that genetics, if properly employed, can allow the cell to tell us important, and completely unexpected, things. Perhaps slightly less obvious, in these days of almost formulaic professionalism, it is important to find a way to give young assistant professors space and funds to carry out innovative research that is a little outside the mainstream.

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