A Conversation with Tim Stearns

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Tim Stearns is the Frank Lee and Carol Hall Professor and a Professor of Genetics at Stanford University.

Jan Witkowski: What are some of the key points about centriole structure and regulation that are of research interest at the moment?

Dr. Stearns: The centrosomes are segregated in mitosis and that is one of the interesting things about them. If you look through history, the realization that they are important organelles in cells happened more than a hundred years ago, but a modern understanding of what they do and how you ensure that each cell gets one at the beginning of the cell cycle has only occurred more recently. They, like chromosomes, are segregated on the mitotic spindle. So, by analogy to chromosomes, that means they have to be duplicated exactly once per cell cycle. The duplication and the segregation of centrosomes and the centrioles within them is really something that you can think about in parallel with thinking about chromosomes.

Jan Witkowski: A centriole looks almost as though it buds off from... I was going to say its parent, but that's not right. The original?

Dr. Stearns: We do use the word "mother," that's true.

Jan Witkowski: How does that process happen? It comes off at right angles.

Dr. Stearns: It's really quite remarkable. For DNA, we've known for a long time—and the original notion of this came from the Watson and Crick double-strand helix—that you replicate DNA by pulling it apart and then use those strands as templates to create new strands. For the centriole, it's been a mystery as to how it duplicates because you don't have that obvious mechanism that comes from the structure. What you do have is the centriole—which is a proteinaceous structure, not nucleic acid—and it has this very characteristic structure, the centriole, which has this beautiful ninefold symmetry and other proteins attach to that. That duplicates just like chromosomes, once every cell cycle.

As you say, it looks somewhat like a new centriole grows as a bud on the side of an existing one. What does happen is that when you initiate centriole formation in a cell in the G₁ phase of the cell cycle, each centriole grows exactly one from its side. The new one grows at a right

angle from the side of the existing one. The mother centriole would be the existing one and a new daughter grows from the side of the mother. That's true in many organisms, so it's really a quite well conserved aspect of how duplication works. And yet, why it works that way, why you only form one new centriole on the side of a mother is still not clear. Even though we know a lot about the molecules involved in duplication, the reason it's constrained to occur exactly there and the way you constrain it to occurring exactly once, are still things that are active areas of work.

Jan Witkowski: What are the molecules that make up a centriole?

Dr. Stearns: The structure of the centriole itself, when you look at the classical image that you can find in textbooks or on Google Images, is this beautiful ninefold symmetric microtubule-based structure, where the microtubules are in triplets. This is unusual. You only find these triplets where there's an A tubule and then a B and a C tubule that grow on the side of the A tubule. The centriole is the only place you find that. Both the ninefold symmetry and having these triplets are things that really define the centriole.

The ninefold symmetry comes from a protein called SAS-6. There's been a lot of really quite beautiful work on this that shows that there's intrinsic behavior of the protein that results in that ninefold symmetry. The SAS-6 protein forms ninefold symmetric oligomers both in vitro and, presumably, in vivo and that's the basis for this ninefold symmetry of the centriole, which is absolutely conserved from all eukaryotes that have centrioles, and presumably in the last common ancestor of all eukaryotes. Those microtubules that this cartwheel that SAS-6 forms are attached to are formed from α - and β -tubulin just like all microtubules in the cell, except that, again, you never find triplet microtubules in any other setting.

For a long time it's been thought that there must be other proteins: δ -tubulin and ϵ -tubulin are members of the tubulin family but are not the canonical members of the ones that make microtubules, and the specialized tubulin, γ -tubulin, that nucleates microtubules, or caps them at least. These are different.

Jan Witkowski: The A, B, and C tubules use the same tubulin molecules?

Dr. Stearns: As regular microtubules, yes. The bulk of those A, B, and the C tubules are made up of α - and β -tubulin, which make this heterodimer that polymerizes to make microtubules. It seems that those centriolar microtubules in this triplet form are made largely of α - and β -tubulin. I say “largely” just to leave open the possibility that there’s something else that might make the triplet microtubules special.

Jan Witkowski: Why don’t they go on to make a quadruplet?

Dr. Stearns: That is a great question. Why is it limited to being a triplet? That is not clear. It’s not clear why they don’t ever go beyond being triplets.

Jan Witkowski: All the things that link the nine, the bundles to keep them in a cylindrical form: Are there things between those triplets?

Dr. Stearns: Yes. Part of what keeps the structure intact is this cartwheel that you see when you do electron microscopy; that’s really the only way you can see it in its full glory in terms of its structure. That’s when you see the ninefold symmetry of this cartwheel at the center of the centriole. Again, there are spokes that radiate out from that and they contact these microtubules that are arranged in ninefold symmetry. Other proteins that are part of that have been seen in the electron microscope but we don’t know the identity of them. There’s something called the A–C linker, which links adjoining triplet microtubules, but no component has ever been identified for those yet.

Jan Witkowski: You’re using genetic tools to dissect structure.

Dr. Stearns: Right. It’s one of the revolutions of cell biology: the ability, using CRISPR–Cas9, to manipulate the genomes of mammalian cells. This ability really started with zinc fingers, which were hard to use and then TALENs, which were easier. But now CRISPR–Cas9 makes it tremendously easy to manipulate the genome of mammalian cells. Not as easy as many people think. If you’re interested in turning mammalian cells into the equivalent of a yeast cell, that’s not really feasible, but this ability to make mutations and then assess the phenotype is key to modern cell biology.

Jan Witkowski: How have you been using CRISPR–Cas9 in your work?

Dr. Stearns: These proteins, δ -tubulin and ϵ -tubulin, which are variant members of the tubulin family are not in all eukaryotes, but they’re in most that have centrioles and cilia. We have been working on these proteins for a long time. We identified them—and others did too—in the late ’90s. Susan Dutcher first identified δ -tubulin and then we identified ϵ -tubulin and more recently we, and others, identified ζ -tubulin: the last member of the tubulin superfamily. I can say that conclusively because we have a lot of sequenced genomes, so it’s clear that there are no other

members of the tubulin superfamily. Because of the conservation of them, we know that the last common ancestor of eukaryotes had all six of these. Some have been lost in various branches of the eukaryotic tree, but if you look at human cells, we have α , β , γ —which all eukaryotes have—and then we have δ - and ϵ -tubulin.

We’ve known this for a long time. The limitation has been, how do you determine the function of proteins in mammalian cells without the ability to manipulate the genome? RNAi, of course, was one of the tools that came along and made some forms of manipulation of gene expression possible. We tried that with δ - and ϵ -tubulin but it never worked very well. Our thought about that is that there’s really a very small number of molecules of these proteins, such that depleting the RNA, even to the levels you typically get with good RNAi, was never sufficient to yield a phenotype. Now, we can make real deletions using Cas9 that allow one to have a defined genotype with a predicted null mutation homozygous form and then assess the phenotype of the cells.

Jan Witkowski: When you knock out these, what happens to the cells? First, what happens to the centriole? Then what’s the effect on the cell?

Dr. Stearns: Using Cas9, we tried hard to knock out both δ -tubulin and ϵ -tubulin and could never identify cell lines where they were clearly knocked out. It turns out that the reason for that is because cells that have a problem with their centrioles, if they are p53-plus, p53 senses a problem and the cells arrest in G₁ of the cell cycle. We didn’t realize at the beginning of our experiment that that was true, but we did soon afterward from the work of others. Once we started doing the experiments in p53-minus cell lines, then we could easily get null mutations. Right away, that hinted that there was going to be something wrong with centrioles that was being sensed by p53. Sure enough, when we looked closely at the phenotype of these mutant cell lines—and both the δ -tubulin and ϵ -tubulin null mutants have very similar phenotypes—the phenotype at the level of the centrioles is that the structure of the centrioles that do exist is different. They only have singlet microtubules instead of triplet microtubules. Remember, those triplets are one of the key features of what makes a centriole special. These lack triplet microtubules and only have singlets.

There’s another aspect to it that caught our eye immediately, which was that if you look at a population of cells that are null mutants for these genes, there’s a very interesting distribution of centrioles in these cells. A normal cell has two centrioles in the G₁ phase of the cell cycle. They duplicate prior to mitosis; you have four. Then they segregate on the spindle and you get a daughter cell with two centrioles again. In this case, about half of the cells had zero centrioles: none detectable whatsoever. The other half of the cells had more than four centrioles: five, six, seven, eight. Right away again, that suggested that there was something very odd about centriole duplication in these cells.

We determined that what was going on, is that that half having centrioles and half having no centrioles is what you see when you look at an asynchronous population, with

cells at all stages of the cell cycle. If you look at specific stages of the cell cycle, you see that in G₁ there are zero centrioles. They form in S Phase. They persist through mitosis and then they disintegrate at some point between mitosis and the next interphase. Actually, probably the start of the next interphase.

It's one of the interesting things about centrioles. Just as we talked about the seeming budding that occurs when you grow a new centriole from the side of an existing one, because that's such a common feature of centriole duplication people have long assumed that you had to have an existing centriole to grow a new one. We know that that is not true because it's been shown many different ways that for many cells from animals and other organisms too, you can create new centrioles *de novo* with no preexisting centriole.

Jan Witkowski: So, you've got these two populations in asynchronous cultures, but if you followed a single cell, you'd find that it at one stage had none and at another stage had several?

Dr. Stearns: Exactly. If you look at a single cell as it progressed through the cell cycle, you start with zero, and you would create centrioles *de novo* with no existing centrioles. Those would persist until mitosis but then they would... I use the word "disintegrate," although actually seeing that happen is not something that we've been able to do, but that's our interpretation of the results.

Jan Witkowski: Are there cells that never have any centrioles? Can cells survive and grow and multiply without centrioles?

Dr. Stearns: It is true that centrioles duplicate exactly once per cell cycle and they participate in the mitotic spindle. Because of their close association with the spindle, people had always assumed that they were required to make the spindle. That is clearly not true, because you can force mammalian cells to lose their centrioles, either genetically or now with a small molecule inhibitor of a required kinase, and in both of those cases the cells will be able to grow without centrioles. So, no centrioles, no centrosome, but they can do mitosis. They have to be p53-minus though, because if you don't have centrosomes, there is a problem—probably with mitosis—that p53 senses. If you get rid of p53, the cells can divide just fine with no centrosomes.

Jan Witkowski: p53 having that sort of oversight absolutely makes a lot of sense in terms of trying to avoid chromosomal abnormalities and such.

Dr. Stearns: Right.

Jan Witkowski: You've shown by knocking out these tubulins that they are associated or involved in important aspects of centrioles. Do you know where they are in the centriole?

Dr. Stearns: We've been unable to really get convincing localization of these proteins. It's not for lack of trying. We, and others, have tried to see where in the cell these proteins are. For cell biologists, that's one of the key clues

to what a protein does. It's been difficult either with antibodies or with tagged proteins of various sorts. I think it's because there're very few molecules of these proteins in cells. That's an assessment based not only on failure to localize the proteins but also on this small amount of RNA and the very low level of protein as assessed with antibodies on western blots. If you look at the centriole structure, where these proteins might fit into that where they could help to make or to stabilize the triplet microtubules, there's several ways you could imagine very low-abundance proteins playing critical roles in that structure.

Jan Witkowski: You mentioned that you've not really been able to follow the disintegration or destruction of these centrioles that end. What do you think is going on? Have you tried messing around with the ubiquitination system to see if you can force them through?

Dr. Stearns: We've gotten close to seeing the disintegration. We certainly can see it by light microscopy, but centrioles are very small structures and light microscopy doesn't really get you to the level you need to be to tell what's going on. By electron microscopy, which we'd done in collaboration with Jadranka Loncarek, we can see that as cells enter mitosis the centrioles elongate, even these aberrant centrioles. They don't have triplet microtubules; they only have singlet microtubules so they're narrower, but they do elongate to the full length. In the electron microscope, it's clear that there's something wrong with them. Having singlets only, it seems that they don't recruit some proteins that are parts of what a maturing centriole would have. They have gaps in the structure. We believe that what is resulting in disintegration is the depolymerization of these singlet microtubules. Thus, the triplet microtubules are really required to be stable. That's what provides the long-term stability of these triplet microtubules, which persist for a very long time. These microtubules don't turn over, unlike most microtubules in the cell.

Jan Witkowski: Centrioles are also a part of cilia?

Dr. Stearns: Yes.

Jan Witkowski: Cilia also get reformed after division?

Dr. Stearns: They do. Most cells in our body that are in G₀ phase—so they're doing their thing as a differentiated cell—will have a primary cilium projecting from the surface from the cell. The microtubule component of that cilium, which is the core structure, is contiguous with the centriole, so it grows from the end of the centriole. When one says that the centriole—or it's often called the basal body in this context—is part of the cilium, it literally is. It is part of the microtubule structure of the cilium. That grows every cell cycle after mitosis in a cycling cell and again, is present in most differentiated cells.

Jan Witkowski: Your "funny" ones don't get involved in the cilium?

Dr. Stearns: We've never seen one with a cilium. That, in part, is because we've also seen that they never have these appendages, which are structures that are added onto the

centriole to functionalize it. In the case of making a cilium, they allow it to interact with the plasma membrane. That interaction is critical to creating the ciliary compartment and you have to do that to be able to traffic molecules specifically into the ciliary compartment to make the cilium. These centrioles never have that ability to attach to the membrane and we never see them associated with a cilium, so that's another defect that these centrioles have.

Jan Witkowski: So those cells never develop cilia?

Dr. Stearns: Right. These null cells never develop cilia, which would clearly be a bad thing for the organism because the primary cilium, at least in vertebrates or mammals, is absolutely required for development.

Jan Witkowski: If I could grant you one wish, what would you really like as your next bit of knowledge?

Dr. Stearns: I think the most useful thing would be to know exactly where those proteins are because when you're working at the level of trying to relate protein function to structure as we are, you really need to be able to say where these proteins—which could be structural components of this complex thing, the centriole—are in the structure. That will inform models for how they actually work. Speculatively, without any evidence yet, this linker between the triplet microtubules—the “A–C linker” it's called, because it links the A tubule to the C tubule of the next triplet—could be a structure that these δ - and ϵ -tubulins are either a component of, or required to make. The absence of that structure might explain the instability of the centriole. That's just a hypothesis and we need to have data about that, but the localization of the proteins would be a great start for that.

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