γ-Tubulin complexes in microtubule nucleation and beyond

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ABSTRACT Tremendous progress has been made in understanding the functions of γ-tubulin and, in particular, its role in microtubule nucleation since the publication of its discovery in 1989. The structure of γ-tubulin has been determined, and the components of γ-tubulin complexes have been identified. Significant progress in understanding the structure of the γ-tubulin ring complex and its components has led to a persuasive model for how these complexes nucleate microtubule assembly. At the same time, data have accumulated that γ-tubulin has important but less well understood functions that are not simply a consequence of its function in microtubule nucleation. These include roles in the regulation of plus-end microtubule dynamics, gene regulation, and mitotic and cell cycle regulation. Finally, evidence is emerging that γ-tubulin mutations or alterations of γ-tubulin expression play an important role in certain types of cancer and in other diseases.

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INTRODUCTION

For many years, the identity of components of microtubule-organizing centers (MTOCs) that nucleate microtubule assembly and establish microtubule polarity was a central unanswered question in the field of mitosis and the cytoskeleton. The discovery of γ-tubulin (Oakley and Oakley, 1989), the key finding that allowed this question to be answered, came from a genetic screen in the fungus *Aspergillus nidulans* designed to identify genes important for microtubule function (Weil *et al.*, 1986). The existence of members of the tubulin superfamily beyond the microtubule proteins α- and β-tubulin was completely unexpected at the time, and the discovery of γ-tubulin is a powerful validation of the value of forward genetics, in which well-designed screens let the cell tell us what is important rather than us imposing our preconceived notions on the cell.

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Abbreviations used: γTuRC, γ-tubulin ring complex; γTuSC, γ-tubulin small complex; APC/C, anaphase-promoting complex/cyclosome; GCP, γ-tubulin complex protein; GRIP, γ-tubulin ring protein; MTOC, microtubule organizing center; RNAi, RNA interference; SAC, spindle assembly checkpoint; SPB, spindle pole body.

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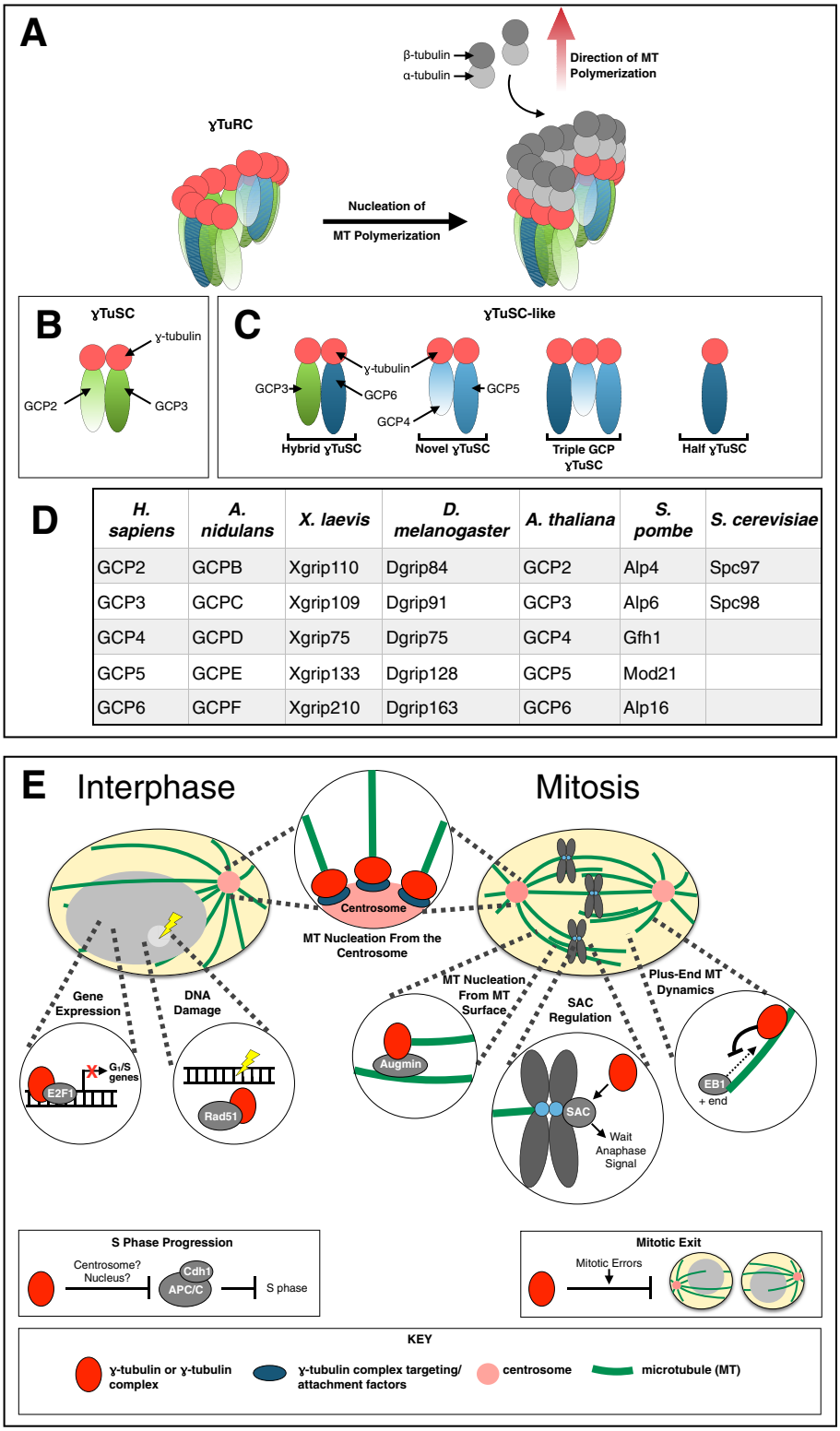
γ-TUBULIN COMPLEXES NUCLEATE MICROTUBULE ASSEMBLY FROM MICROTUBULE-ORGANIZING CENTERS

γ-Tubulin is ubiquitous in eukaryotes, and genome-sequencing projects have revealed that there are one to three γ-tubulin genes in eukaryotic genomes (Findeisen *et al.*, 2014). It localizes to structurally diverse MTOCs and, with few exceptions, is required for microtubule nucleation (reviewed by Job *et al.*, 2003). Ring-shaped structures called γTuRCs (γ-tubulin ring complexes) that contain γ-tubulin and associated proteins nucleate microtubule assembly in vitro (Zheng *et al.*, 1995) and in vivo (Figure 1, A–D) (reviewed by Teixido-Travesa *et al.*, 2010; Kollman *et al.*, 2011; Lin *et al.*, 2014). Proteins that associate with γ-tubulin have been identified and designated GRIPs (γ-tubulin ring proteins) or GCPs (γ-tubulin complex proteins) (Figure 1D). Five of these proteins, GCP2–6, are structurally related and contain conserved regions called GRIP motifs (Gunawardane *et al.*, 2000; Guillet *et al.*, 2011).

Beyond GCP2–6, γTuRCs contain three additional, more recently discovered, core subunit proteins, MOZART1 (mitotic spindle-organizing protein associated with a ring of γ-tubulin, or GCP9) and MOZART2A and MOZART2B (GCP8A and GCP8B), which are closely related to each other (Hutchins *et al.*, 2010). MOZART1 is highly conserved in eukaryotes, except in *Saccharomyces cerevisiae* and close relatives (Hutchins *et al.*, 2010; Lin *et al.*, 2014), and it is essential for mitotic spindle assembly and function in humans and *Schizosaccharomyces pombe* (Hutchins *et al.*, 2010; Masuda *et al.*, 2013). *Arabidopsis thaliana* has two MOZART1 homologues, Gip1



H. sapiens	A. nidulans	X. laevis	D. melanogaster	A. thaliana	S. pombe	S. cerevisiae
GCP2	GCPB	Xgrip110	Dgrip84	GCP2	Alp4	Spc97
GCP3	GCPC	Xgrip109	Dgrip91	GCP3	Alp6	Spc98
GCP4	GCPD	Xgrip75	Dgrip75	GCP4	Gfh1	
GCP5	GCPE	Xgrip133	Dgrip128	GCP5	Mod21	
GCP6	GCPF	Xgrip210	Dgrip163	GCP6	Alp16	

FIGURE 1: γ-Tubulin or γ-tubulin complexes in microtubule nucleation and beyond. (A) Nucleation of microtubule assembly by a γTuRC. The γTuRC consists of GCPs that bind to γ-tubulin and to each other, forming a complex in which a ring of γ-tubulin molecules effectively mimics the plus end of a microtubule. It forms a pre-existing nucleus for microtubule assembly. At physiological tubulin concentrations, spontaneous assembly of tubulin into microtubules is rare. Instead, almost all microtubule growth occurs from preformed nucleating structures, principally the γTuRC. (B) In *S. cerevisiae* only two GCPs exist, Spc97 and Spc98. They assemble with γ-tubulin to form γTuSCs. (C) The finding that GCP2–6 all bind to γ-tubulin raises the possibility that GCPs and γ-tubulin may assemble into alternative γTuSC-like structures (Kollman *et al.*, 2011) that assemble along with γTuSCs to form the γTuRC. (D) Designations for GCPs in various organisms in which they have been studied extensively. (E) Functions of γ-tubulin. γ-Tubulin complexes nucleate microtubules from the centrosome and other MTOCs in both interphase and mitosis or from microtubule surfaces in mitosis. In interphase and mitosis, γ-tubulin has been shown to regulate microtubule plus-end dynamics. In mitosis, it plays a role in the spindle assembly checkpoint (SAC) and the control of mitotic exit. In interphase, γ-tubulin plays an important role in inhibiting APC/C^{Cdh1}, thereby promoting the transition from the G_1 to the S phase. It also appears to regulate E2F1-mediated gene expression and complexes with Rad51, suggesting a role in the Rad51-mediated DNA damage response.

and Gip2, that interact with GCP3. They are not essential individually, but Gip1/2 double mutants are embryonic lethal (Nakamura *et al.*, 2012). MOZART2A and MOZART2B are apparently restricted to vertebrates, in which they play a role in recruitment of the γTuRC to the centrosome (Teixido-Travesa *et al.*, 2010; Lin *et al.*, 2014). In addition to the core γTuRC components, various phyla have proteins, unrelated in sequence to GCP2–9, that are involved in targeting γ-tubulin complexes to MTOCs (reviewed extensively by Lin *et al.*, 2014).

THE STRUCTURE OF THE γTURC SUGGESTS A MECHANISM FOR MICROTUBULE NUCLEATION

High-resolution structures of human γ-tubulin (Aldaz *et al.*, 2005; Rice *et al.*, 2008) and GCP4 (Guillet *et al.*, 2011) and lower-resolution structures of small γ-tubulin complexes (γTuSCs, containing two molecules of γ-tubulin and one molecule each of GCP2 and 3) from *S. cerevisiae* (Kollman *et al.*, 2008, 2010) have been determined, and these and other findings have led to a persuasive model for the structure of the γTuRC (Kollman *et al.*, 2011). In this model, GCP2–6 each binds directly to γ-tubulin and assemble into a structure that contains 13 γ-tubulin molecules (perhaps containing five γTuSCs and one molecule each of GCP4-6 bound to γ-tubulin; Figure 1A). GCP3 contains a hinge region, and in the current model, movement about this hinge would alter the positioning of γ-tubulin molecules. In one position, the γ-tubulin molecules would be too far apart to nucleate microtubule assembly, but in the other position, the straight position, they would have exactly the same spacing as microtubule protofilaments and would form a perfect template for microtubule assembly.

Control of the conformation of GCP3 could therefore regulate the ability of the γTuRC to nucleate microtubule assembly. Important unanswered questions in this area are the exact arrangement of GCP2–6 in the γTuRC and the positioning of MOZART1, 2A, and 2B in the complex. The hinge model, although persuasive, needs to be tested rigorously, and if it is correct, it raises the question of what controls the rotation of GCP3 around its hinge.

γ-TUBULIN COMPLEXES NUCLEATE MICROTUBULES FROM THE SIDES OF EXISTING MICROTUBULES

Although microtubule nucleation from MTOCs is extremely important, in many phyla MTOCs are not apparent. Even in vertebrate mitosis, centrosomes are not always required for the formation of a functional spindle (Khodjakov *et al.*, 2000), and when centrosomes are present, many microtubules may be nucleated independent of the centrosome. Similarly MTOC-independent nucleation of cytoplasmic microtubules occurs in many phyla.

Murata *et al.* (2005) showed convincingly that cortical microtubules in higher plant cells are nucleated from the sides of existing microtubules at a characteristic angle of 42° with respect to existing microtubules, that γ-tubulin is at the branch points, and that lateral microtubule nucleation is γ-tubulin dependent. Similarly, Janson *et al.* (2005) demonstrated that microtubules are nucleated from γ-tubulin complexes at the sides of cytoplasmic microtubules in *S. pombe*. These data established the principle that γ-tubulin complexes function in nucleation of microtubules from the sides of existing microtubules. Data obtained over the intervening decade have identified the augmin complex as a key player in lateral microtubule nucleation.

Augmin is an eight-subunit complex that is important for localizing γ-tubulin to mitotic spindles and for centrosome-independent microtubule generation in mitotic and meiotic spindles (Goshima *et al.*, 2008; Ho *et al.*, 2011; Petry *et al.*, 2011; Hsia *et al.*, 2014). In higher plants, augmin colocalizes with GCPs, and they are corecruited to cortical microtubules. Knockdown of augmin with a microRNA reduces branching lateral nucleation of microtubules (Liu *et al.*, 2014). In *Xenopus* extracts, augmin and γTuRCs along with the microtubule assembly factor TPX2 and the GTP-bound RanGTPase nucleate microtubule assembly, forming fan-like structures consistent with the possibility that the microtubules are in branched arrays (Petry *et al.*, 2013). Microtubule nucleation persists if augmin or TPX2 are depleted, but fan-like arrays do not form. Monitoring the plus-end microtubule-tracking protein EB-1 revealed that new microtubules are nucleated from the sides of existing microtubules and that the newly nucleated microtubules have the same polarity as the microtubules from which they are nucleated. Such lateral nucleation of microtubule assembly in a mitotic apparatus would result in the proliferation of spindle microtubules but, importantly, would not disrupt the polarity of microtubules in the spindle. These and related findings support a persuasive although not completely proven model that the augmin complex binds to microtubule walls and recruits the γTuRC through NEDD1 or other proteins to initiate new microtubule nucleation (Uehara *et al.*, 2009; Zhu *et al.*, 2009; Johmura *et al.*, 2011). The fact that augmin apparently plays a role in lateral microtubule nucleation in both plants and animal suggests that augmin-mediated lateral microtubule nucleation is widespread in eukaryotes. However, many organisms do not have homologues of all augmin subunits (Lin *et al.*, 2014), and the fact that the polarity of microtubules nucleated from the sides of microtubules in *S. pombe* is opposite to that of augmin-mediated nucleation (Janson *et al.*, 2005) suggests there are at least two lateral nucleation mechanisms.

The theme that has emerged from two decades of research is that γ-tubulin complexes are almost universally involved in microtubule nucleation. Mechanisms have evolved, however, to control and target these complexes, such that microtubule nucleation occurs when and where it is needed.

γ-TUBULIN BEYOND MICROTUBULE NUCLEATION

The traditional view has been that each protein has a single function, but increasingly, proteins have been shown to be multifunctional. In this regard, increasing evidence indicates that γ-tubulin has functions beyond microtubule nucleation (Cuschieri *et al.*, 2007). In many ways, this makes sense, because γ-tubulin is at the hub of the microtubule cytoskeleton and, along with other MTOC components, is in a unique position to receive and send signal molecules transported by microtubule motors. The problem with interpreting some of these data, however, is that, because γ-tubulin has a critical role in mitosis and disruption of mitosis has many consequences, it is difficult to distinguish putative non–microtubule-nucleation functions of γ-tubulin from consequences of mitotic failure or alteration of microtubule nucleation. Nevertheless, we believe that there is ample evidence that γ-tubulin has important functions beyond microtubule nucleation (summarized in Figure 1E).

γ-TUBULIN PLAYS A ROLE IN THE REGULATION OF MICROTUBULE PLUS-END DYNAMICS

There have been repeated reports from multiple phyla that mutations or deficiencies in γ-tubulin or GCPs alter plus-end microtubule dynamics (Paluh *et al.*, 2000; Vogel *et al.*, 2001; Zimmerman and Chang, 2005; Bouissou *et al.*, 2009) and that γ-tubulin alters the distribution of the plus end–tracking protein Bim1 in *S. cerevisiae* (Cuschieri *et al.*, 2006) and its homologue, EB1, in *Drosophila* (Bouissou *et al.*, 2014). This is surprising, since γ-tubulin is most obviously located at the minus end. Two non–mutually exclusive explanations have been proposed. Considering that there is constant bidirectional transport along microtubules, one idea is that γ-tubulin complexes at MTOCs could bind catastrophe or rescue factors or the motor molecules that transport them. This would facilitate loading of molecules that affect microtubule dynamics onto motor molecules, thereby affecting their transport to microtubule plus ends and, consequently, affecting microtubule dynamics at plus ends. Consistent with this idea, γ-tubulin mutations alter binding of motor proteins and/or microtubule stability factors (Vogel *et al.*, 2001; Zimmerman and Chang, 2005; Cuschieri *et al.*, 2006). For example, some γ-tubulin mutations alter the binding of kinesin-14 and kinesin-5 to γ-tubulin complexes, while others alter the interaction of cytoplasmic dynein with spindle pole bodies (SPBs; Li *et al.*, 2005; Rodriguez *et al.*, 2008; Olmsted *et al.*, 2014), although the binding of type 5 and 14 kinesins may be involved in regulating microtubule nucleation rather than plus-end microtubule dynamics (Olmsted *et al.*, 2014).

The second proposed explanation comes from work in *Drosophila*, in which γTuRC proteins localize along astral microtubules and are proposed to act as rescue factors that arrest microtubule disassembly (Bouissou *et al.*, 2009, 2014). It is worth noting that the proposed rescue activity of γTuRCs distal to MTOCs might not be intrinsic to the γTuRC but a function of its binding to rescue factors, as discussed above. Another possibility that is unfortunately difficult to test is that γ-tubulin mutations alter the microtubule lattice and thereby alter microtubule dynamics.

γ-TUBULIN FUNCTIONS IN THE REGULATION OF MITOSIS AND THE CELL CYCLE

There have also been persuasive reports of perturbation of the regulation of mitotic exit by alterations of γ-tubulin or GCPs. Alanine-scanning mutants of human γ-tubulin expressed as the sole γ-tubulin

in *S. pombe* allowed cells to go through anaphase and cytokinesis when spindle formation was disrupted (Hendrickson *et al.*, 2001). Mutations in the GCP4 and GCP5 homologues of *S. pombe* caused a failure of mitotic arrest in the presence of the antimicrotubule agent thiabendazole (Vardy and Toda, 2000). Similarly, a γ-tubulin mutation in *A. nidulans* caused mitotic exit before successful completion of mitosis in a strain in which the establishment of spindle bipolarity was delayed by a type 14 kinesin deletion (Prigozhina *et al.*, 2001). Another allele caused a premature mitotic exit even when the microtubule cytoskeleton was completely disassembled with benomyl (Prigozhina *et al.*, 2004). Likewise, RNA interference (RNAi) of Dgrip84 (GCP2) in *Drosophila* caused an untimely mitotic exit in the presence of colchicine (Colombie *et al.*, 2006). Thus genetic or RNAi perturbations of γ-tubulin or GCPs in three organisms and four laboratories all point to a microtubule nucleation-independent role for γ-tubulin complexes in the control of mitotic exit.

A possibly related area in which results from several labs have indicated a non–microtubule nucleation function for γ-tubulin is coordination of mitotic events and/or the spindle assembly checkpoint (SAC). In a systematic alanine mutagenesis screen, a number of γ-tubulin mutants were shown to cause premature anaphase entry and defective cytokinesis, suggesting that γ-tubulin regulates the SAC and orderly mitotic exit (Hendrickson *et al.*, 2001). An *A. nidulans* γ-tubulin allele caused late mitotic events (chromosomal disjunction, spindle elongation, and mitotic exit) to become disordered (Prigozhina *et al.*, 2004). The failure of chromosomal disjunction in this instance was not due to lack of microtubule assembly or force generation, because chromosomes were often stretched dramatically in anaphase, even though they did not disjoin. With respect to mitotic regulation and the SAC, it is worth noting that there are reports that the SAC/mitotic regulatory proteins CDC20 and BubR1 are in a complex with γ-tubulin in mammalian cells (Muller *et al.*, 2006) and that Mad2 binds to Alp4p (GCP2) in *S. pombe* (Mayer *et al.*, 2006).

Finally, there is incontrovertible evidence that γ-tubulin plays a role in the regulation of cell cycle progression in interphase. In *A. nidulans*, a γ-tubulin allele, *mip*AD159, causes failure of inactivation of the anaphase-promoting complex/cyclosome (APC/C) at the G_1/S boundary (Nayak *et al.*, 2010). This failure correlates with failure of the APC/C activator Cdh1 to dissociate from the SPB, and it can be overridden by deletion of Cdh1 (Edgerton-Morgan and Oakley, 2012). Importantly, this failure occurs if microtubules are present or if they are completely depolymerized, so it is not a microtubule nucleation phenomenon (Nayak *et. al.*, 2010). This allele also causes mislocalization of the SAC proteins Bub1/R1 (*A. nidulans* has a single gene with functional domains of Bub1 and BubR1) and Mps1, thereby abrogating the SAC (Edgerton *et al.*, 2015). Note also that analyses of an *alp4* (GCP2) mutation indicate Alp4 has an essential function in G_1 in *S. pombe* (Vardy and Toda, 2000). This mutation, moreover, can cause septation, even when mitosis is arrested, by allowing inappropriate recruitment of the Sid1 kinase to the SPB (Vardy *et al.*, 2002). There are also reports that γ-tubulin modulates the activity of E2F transcription factors, which control the expression of a number of genes that are necessary for DNA replication and centrosome duplication (Hoog *et al.*, 2011; Ehlen *et al.*, 2012). The key challenge in understanding the various nonnucleation functions of γ-tubulin will be to move from phenomenology to molecular mechanism.

γ-TUBULIN IN DISEASE

Although the functional significance is not yet clear, there is strong and intriguing evidence of the association of γ-tubulin with tumor suppressor proteins, particularly those involved in DNA repair. It binds to Rad51 in nuclei in response to DNA damage (Lesca *et al.*, 2005) and coimmunoprecipitates with ATR, BRCA1, and C53 from nuclei (Zhang *et al.*, 2007; Hubert *et al.*, 2011; Horejsi *et al.*, 2012). BRCA1/BARD1 binds to and monoubiquitinates γ-tubulin, and there is evidence that this ubiquitination is important in the regulation of centrosome number (Hsu *et al.*, 2001; Starita *et al.*, 2004), a particularly interesting finding, since centrosome amplification is important to cancer progression.

γ-Tubulin expression or localization pattern is altered in a variety of cancers, including some multiple myelomas, non–small cell lung cancers (Maounis *et al.*, 2012; Dementyeva *et al.*, 2013), ductal hyperplasia and breast cancer (Niu *et al.*, 2009; Cho *et al.*, 2010), gliomas and glioblastoma cell lines (Katsetos *et al.*, 2006), and medulloblastomas and medulloblastoma cell lines (Caracciolo *et al.*, 2010). Spontaneous mutations in one of the two human γ-tubulin genes, *TUBG1*, are associated with lissencephaly and microcephaly (Poirier *et al.*, 2013; Bahi-Buisson *et al.*, 2014). Further effort devoted to studies of both the microtubule nucleation-dependent and nucleation-independent functions of γ-tubulin in different cell and tissue contexts should shed light on the relevance of this cytoskeleton protein in health and disease.

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