

# Microtubule affinity-regulating kinase 4 (MARK4) is a component of the ectoplasmic specialization in the rat testis

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**Keywords:** testis, spermatogenesis, seminiferous epithelial cycle, microtubule, actin filament, MARK4, ectoplasmic specialization, spermiogenesis, Sertoli cells, germ cells, cell adhesion

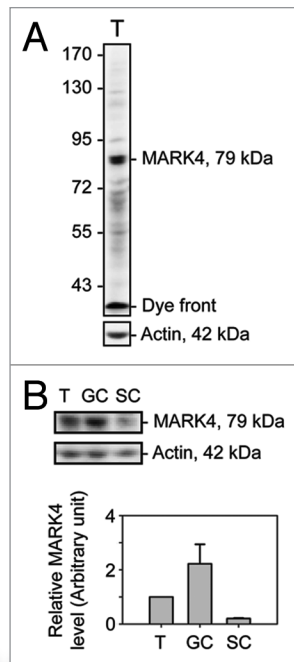
During the seminiferous epithelial cycle of spermatogenesis, the ectoplasmic specialization (ES, a testis-specific adherens junction, AJ, type) maintains the polarity of elongating/elongated spermatids and confers adhesion to Sertoli cells in the seminiferous epithelium, and known as the apical ES. On the other hand, the ES is also found at the Sertoli-Sertoli cell interface at the blood-testis barrier (BTB) known as basal ES, which together with the tight junction (TJ), maintains Sertoli cell polarity and adhesion, creating a functional barrier that limits paracellular transport of substances across the BTB. However, the apical and basal ES are segregated and restricted to the adluminal compartment and the BTB, respectively. During the transit of preleptotene spermatocytes across the BTB and the release of sperm at spermiation at stage VIII of the seminiferous epithelial cycle, both the apical and basal ES undergo extensive restructuring to facilitate cell movement at these sites. The regulation of these events, in particular their coordination, remains unclear. Studies in other epithelia have shown that the tubulin cytoskeleton is intimately related to cell movement, and MARK [microtubule-associated protein (MAP)/microtubule affinity-regulating kinase] family kinases are crucial regulators of tubulin cytoskeleton stability. Herein MARK4, the predominant member of the MARK protein family in the testis, was shown to be expressed by both Sertoli and germ cells. MARK4 was also detected at the apical and basal ES, displaying highly restrictive spatiotemporal expression at these sites, as well as co-localizing with markers of the apical and basal ES. The expression of MARK4 was found to be stage-specific during the epithelial cycle, structurally associating with  $\alpha$ -tubulin and the desmosomal adaptor plakophilin-2, but not with actin-based BTB proteins occludin,  $\beta$ -catenin and Eps8 (epidermal growth factor receptor pathway substrate 8, an actin bundling and barbed end capping protein). More importantly, it was shown that the expression of MARK4 tightly associated with the integrity of the apical ES because a diminished expression of MARK4 associated with apical ES disruption that led to the detachment of elongating/elongated spermatids from the epithelium. These findings thus illustrate that the integrity of apical ES, an actin-based and testis-specific AJ, is dependent not only on the actin filament network, but also on the tubulin-based cytoskeleton.

## Introduction

Polarity-inducing kinases of the MARK [microtubule-associated protein (MAP)/microtubule affinity-regulating kinase] family are Ser/Thr protein kinases initially identified in *Caenorhabditis elegans*, *Drosophila melanogaster* and *Saccharomyces cerevisiae*, and they are related to the PAR (partitioning-defective) gene products.<sup>1-3</sup> MARK is also known as Par-1 in nematodes and fruit flies.<sup>4</sup> Four MARK isoforms, namely MARK1, 2, 3 and 4, have been identified in humans to date, which form a subfamily of the calcium/calmodulin-dependent protein kinase (CAMP) group of kinases.<sup>5</sup> MARK4 is the human ortholog of Par-1, and

unlike Par3 and Par6 which are restricted to the apical region of epithelial cells, MARK4 is located mostly at the basal region of cells, conferring apico-basal polarity during cellular events such as embryonic development,<sup>4,6</sup> illustrating its significance in cell polarity. MAPKs were first shown to be capable of phosphorylating tau and related microtubule-associated proteins (MAPs) at Ser residues within KXGS motifs present in microtubule binding repeats, triggering the disruption of the microtubule network.<sup>7</sup> For instance, it is known that tau [Note: Tau proteins are products of alternative splicing from a single gene known as MAPT<sup>8,9</sup> (microtubule-associated protein tau), which are crucial to stabilize microtubules in cells, such as neurons] is highly soluble

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Submitted: 04/02/12; Revised: 04/28/12; Accepted: 05/02/12  
<http://dx.doi.org/10.4161/spmg.20724>



**Figure 1.** A study to characterize the anti-MARK4 antibody and the cellular distribution of MARK4 in the rat testis. The specificity of the anti-MARK4 antibody was demonstrated by immunoblotting using ~60  $\mu$ g protein from lysates of adult rat testes using a 6.5% 7 SDS-polyacrylamide gel, with actin as a loading control (A). A prominent band with an electrophoretic mobility of ~79 kDa corresponding to the predicted apparent Mr of MARK4 was detected by immunoblotting. The relative level of MARK4 in lysates from adult rat testes (T), germ cells (GC) isolated from 90-d-old rat testes and cultured in vitro for ~16 h, and Sertoli cells (SC) isolated from 20 d-old rat testes and cultured in vitro for ~4 d were shown in (B), and the composite data were shown in a histogram in (C). Each bar = mean  $\pm$  SD of n = 3 independent experiments.

undernormal physiological conditions. Hyperphosphorylation of tau by MAPK (mitogen activated protein kinase) is one of the initial events for the abnormal/pathological aggregation of insoluble tau to neurofibrillary tangles.<sup>10</sup> Coupled with amyloid plaques, these are the two hallmarks of Alzheimer disease, since they lead to the degradation of neurons in the brain and result in Alzheimer disease. Other studies have shown the substrates of MARKs to include many cellular regulatory proteins, such as cell cycle regulating phosphatase Cdc25, class IIa histone deacetylases, MAPK, scaffolding protein KSR1 (kinase suppressor of Ras 1), and desmosomal armadillo protein plakophilin 2 (PKP2).<sup>11-13</sup> Thus, since the early findings on the effects of MARKs on tau protein phosphorylation in 1997,<sup>7</sup> which illustrated their role in microtubule dynamics, MARKs are now known to be involved in regulating a diverse array of cellular processes such as cell cycle, cell polarization, neuronal function and cell signaling.<sup>1-3</sup> In this context, it is of interest to note that recent studies have shown that a simultaneous knockdown of connexin 43 (Cx43) and PKP2 by RNAi, which are components of the gap junction (GJ) and the desmosome at the BTB, affects distribution of TJ-proteins at the BTB (e.g., occludin, ZO-1), causing their mis-localization, thereby perturbing the BTB integrity.<sup>14</sup> Since PKP2 is a substrate of MAPK4,<sup>13</sup> MAPK4 may play a critical

role in BTB dynamics via its effects on the gap junction (GJ) and desmosome, such as via an interaction with PKP2, at the BTB during spermatogenesis.

During the seminiferous epithelial cycle of spermatogenesis, highly polarized Sertoli cells and spermatids arising from meiosis II undergo spermiogenesis.<sup>15,16</sup> For instance, the tight junction (TJ), basal ES, GJ and desmosome that constitute the BTB are restricted near the basement membrane, and these junctions segregate the seminiferous epithelium into the basal and the adluminal compartments.<sup>17,18</sup> Additionally, during spermiogenesis, spermatids that derive from meiosis II undergo extensive morphological transformations via 19 steps in rats (16 in mice) to form elongated spermatids (spermatozoa).<sup>19,20</sup> Beginning from step 8 spermatids that appear at stage VIII of the cycle, the apical ES forms, and its function is to anchor step 8–19 spermatids onto the Sertoli cell so that germ cells can obtain structural and nourishment. Once the apical ES appears, it remains as the only anchoring device that persists throughout spermiogenesis until its degeneration prior to spermiation,<sup>16,21</sup> illustrating that the ES is the critical ultrastructure that confers polarity to developing spermatids (apical ES) and Sertoli cells (basal ES). Both the apical and basal ES are typified by the presence of conspicuous actin filament bundles that lie perpendicular to the apposing plasma membranes of the Sertoli-spermatid and Sertoli-Sertoli cell interface, respectively, and they are sandwiched in-between the cisternae of endoplasmic reticulum and the cell membrane,<sup>16,17,21</sup> illustrating the significance of the actin filament network to ES function. Moreover, the apical ES is considered to be one of the strongest adhesive junctions, significantly stronger than the desmosome<sup>22</sup>, which is probably due to the unusual actin filament network at the site,<sup>23</sup> yet spermatids move progressively up-and-down the epithelium at spermiogenesis during the epithelial cycle, this thus requires the presence of microtubules at the apical ES to serve as a “track” for spermatid migration.<sup>21,24</sup> However, there is no study in the literature reporting the biology, maintenance and regulation of the microtubule network at the ES. We thus thought it pertinent to examine the localization of MARK4 at the apical and basal ES and its likely interacting partners at these sites as our initial attempt to understand the role of microtubules in ES function. Based on the restrictive temporal and spatial expression of MARK4 in the seminiferous epithelium during the epithelial cycle, MARK4 appears to be a crucial protein that stabilizes microtubules in Sertoli cells at the ES. This is the subject of this report.

## Results

**Stage-specific localization of MARK4 at the apical ES and BTB in the seminiferous epithelium of adult rat testes.** Using a specific antibody against MARK4 (Fig. 1A and Table 1), MARK4 was detected in the testis by immunoblotting (Fig. 1A–C), and it was found to be more abundant in germ cells than in Sertoli cells. These findings were consistent with results from immunofluorescence microscopy experiments, in which MARK4 localized prominently to spermatogonia and early spermatocytes throughout the seminiferous epithelial cycle (Fig. 2). However,

**Table 1.** Antibodies used for different experiments in this report

Antibody*	Vendor	Catalog number	Application	Working dilution for IB, (IF), IHC, Co-IP
Rabbit anti-MARK4	Abcam	ab32529	IB, IF, Co-IP	1:1,000, (1:100), 1:150
Rabbit anti-MARK1–4	Abcam	ab74131	IHC	1:100
Rabbit anti-MARK4	Cell Signaling Technology	4834	IB	1:500
Mouse anti-N-cadherin	Invitrogen	33–3900	IF	(1:100)
Mouse anti-ZO-1	Invitrogen	33–9188	IF	(1:100)
Mouse anti-Arp3	Sigma-Aldrich	A5979	IF	(1:100)
Mouse anti-laminin $\alpha$ 2	EMD Millipore	MAB1922Z	IF	(1:100)
Rabbit anti- $\beta$ -catenin	Invitrogen	71–2700	Co-IP	1:50
Rabbit anti-occludin	Invitrogen	71–1500	IB	1:250
Mouse anti-Eps8	BD Biosciences	610143	IB	1:5000
Goat anti-plakophilin-2	Santa Cruz Biotechnology	sc-18976	Co-IP	1:400
Goat anti-actin	Santa Cruz Biotechnology	sc-1616	IB	1:300
Rabbit anti- $\beta$ -tubulin	Abcam	ab6046	Co-IP	1:150

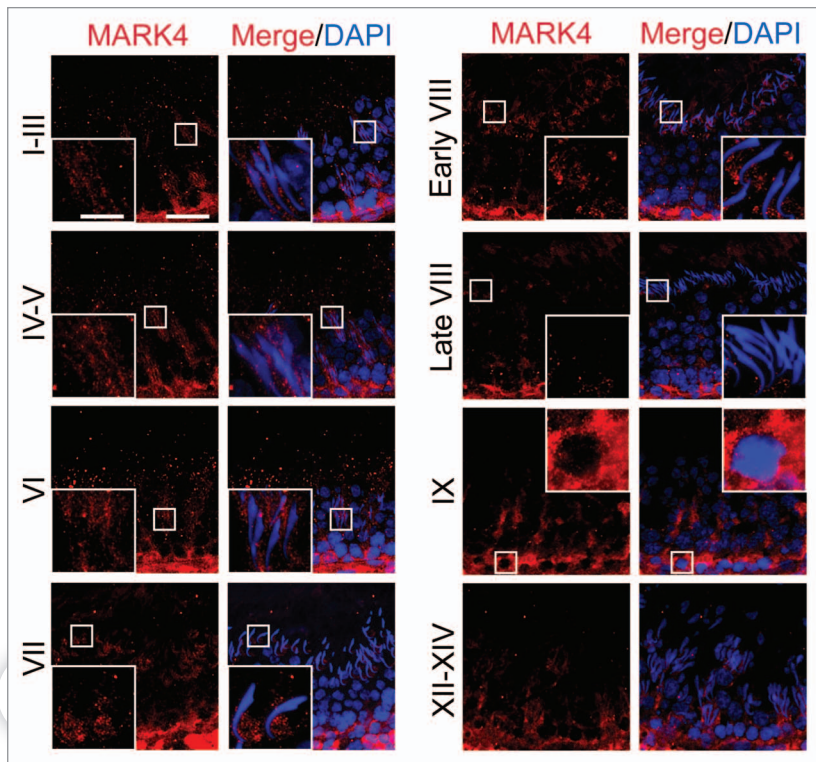
\*Antibodies used in this study cross-reacted with the corresponding proteins in rats as indicated by the manufacturers. IB, immunoblotting; IF, immunofluorescence microscopy; IHC, immunohistochemistry; Co-IP, co-immunoprecipitation.

the expression of MARK4 by more advanced germ cells, such as round spermatids and elongating/elongated spermatids, was found to be highly stage-specific during the epithelial cycle (Fig. 2). The localization of MARK4 at the BTB appeared to be stage-specific since its presence at this site was prominent in most stages of the epithelial cycle except for stages XII–XIV when its expression was considerably reduced. Moreover, the intense localization of MARK4 at the site of the BTB, as well as with migrating leptotene spermatocytes, was clearly visible at stage IX of the cycle (Fig. 2). At the apical ES, MARK4 was also expressed stage-specifically, being highest from stage I to early VIII, its level of expression diminished considerably to an almost non-detectable level by late stage VIII, coinciding with the release of sperm at spermiation (Fig. 2). Also, MARK4 displayed unusual changes in its spatiotemporal localization at the apical ES, surrounding the head of elongating spermatids during the epithelial cycle. For instance, MARK4 was found to surround the entire elongating spermatid head from stages I–V. However, its localization began to shift as the cycle progresses, limited almost exclusively to the concave side of the spermatid head. This restrictive localization was clearly visible at stage VII, and at early stage VIII; the pattern of restricted localization at the concave side of the spermatid head gradually vanished, moving away from the site, and became almost completely undetected by late stage VIII (Fig. 2). This restrictive spatiotemporal expression of MARK4 seemingly suggests that this protein may be related to the stability of the ES during spermatogenesis, such as apical ES during spermiogenesis or basal ES at the BTB via its effects in maintaining the tubulin network at both sites. It appears that its virtual “disappearance” at the apical ES correlates with the apical ES degeneration to facilitate the release of sperm at spermiation. The stage-specific and cellular localization of MARK4 at the apical ES and the BTB was further confirmed by immunohistochemistry (IHC) using an anti-MARK1–4 antibody (Fig. 3A–F). It was also shown that MARK4, at 79 kDa, is the predominant form of MARK proteins in the testis, such as in germ cells (Fig. 3A).

Also, the cellular localization of MARK1–4 at the BTB and the apical ES shown in Figure 3C–F vs. Figure 3A (negative control) is consistent with findings using the specific MARK4 antibody shown in Figure 2.

**Co-localization of MARK4 with markers of the hemidesmosome, BTB and apical ES in the seminiferous epithelium.** The prominent localization of MARK4 at the BTB and apical ES in the seminiferous epithelium is further supported by using an antibody against MARK1, 2, 3 and 4 (Fig. 3A–F). However, MARK4 was found to be the most predominant MARK protein among MARK1–4 in the testis since an anti-MARK antibody specifically recognized a consensus stretch of sequence of NH<sub>2</sub>-LDTFC-COOH among MARK1, 2, 3 and 4<sup>25</sup> (see Table 1) was found to detect a prominent 79 kDa band of MARK4 (Fig. 3B vs. 1A). Using this MARK1–4 antibody, members of MARK were found to be restricted to the apical ES and BTB (Fig. 3C–F), however, spermatogonia and spermatocytes were also stained positive by this antibody as shown in Figure 3C and E. Recent studies have shown that there is a functional axis known as the “apical ES-BTB-hemidesmosome/basement membrane” axis,<sup>16,26</sup> so we next examined if MARK4 can be a likely regulator in this axis by first examining its localization with corresponding marker proteins along this axis (Fig. 4). It was found that MARK4 co-localized with laminin  $\alpha$ 2 (a marker of hemidesmosome),<sup>26</sup> ZO-1 (a TJ adaptor) and N-cadherin (a basal ES integral membrane protein) (both are markers of the BTB),<sup>16,27</sup> and Arp3 (actin-related protein 3, which together with Arp2 forms the Arp2/3 protein complex that regulates branched actin polymerization),<sup>28,29</sup> implicating its likely involvement in regulating this functional axis via its effects to maintain tubulin cytoskeleton stability,<sup>1,30,31</sup> such as at the ES.

**Structural interactions between MARK4 and proteins at the apical ES and the BTB.** To identify the interacting partners of MARK4, co-immunoprecipitation (Co-IP) using lysates of rat testes was performed (Fig. 5). It was found that MARK4 structurally associated with  $\beta$ -tubulin (Fig. 5), which together with



**Figure 2.** Stage-specific and restrictive temporal and spatial expression of MARK4 in the seminiferous epithelium during the seminiferous epithelial cycle of spermatogenesis. Frozen cross-sections of testes (7  $\mu\text{m}$  in thickness) were obtained with a cryostat at  $-20^{\circ}\text{C}$ , fixed in Bouin's fixative and processed for fluorescence microscopy using an anti-MARK4 antibody (see Table 1).<sup>59,71</sup> Cell nuclei were visualized by DAPI (4',6-diamidino-2-phenylindole) staining. Different stages of the seminiferous epithelial cycle are labeled. MARK4 was detected at the apical ES, BTB and the basement membrane. The expression of MARK4 was found to be stage-specific, with its expression at the BTB being the highest at stages VI–IX. Its expression at the apical ES appeared in stage IX and persisted through early stage VIII, but declined considerably to an almost non-detectable level by late stage VIII. Its localization at the apical ES also changed during the epithelial cycle. For instance, immunoreactive MARK4 was found to surround the entire head of elongating spermatids at stages IV–VI, but its localization was shifted and it was found to be restricted to the concave side of the spermatid head at stage VII, and diminished in early stage VIII. In some micrographs, a selected area is boxed and magnified in the inset. Bar in the first micrograph = 50  $\mu\text{m}$ , which applies to all low magnification micrographs; bar = 20  $\mu\text{m}$  in inset, which also applies to all insets.

$\alpha$ -tubulin are known to constitute microtubules in mammalian cells.<sup>32</sup> MARK4 also interacted with desmosomal armadillo protein plakophilin-2, an adaptor of desmosomes at the BTB, which was shown to form a regulatory protein complex with connexin-43 that modulated protein distribution at the BTB since the simultaneous knockdown of connexin-43 and plakophilin-2 by RNAi impeded the distribution of TJ-proteins (e.g., occludin, ZO-1) at the Sertoli cell BTB, rendering these proteins to redistribute from the Sertoli cell-cell interface into the cell cytosol, destabilizing the TJ-permeability barrier function.<sup>14</sup> However, MARK4 did not structurally interact with TJ- (e.g., occludin), and basal ES- (e.g.,  $\beta$ -catenin, Eps8) proteins at the BTB, or apical ES-protein (e.g., Eps8) (Fig. 5).

**MARK4 supports the integrity of the apical ES via its effects on the tubulin stability.** In order to further define the role of

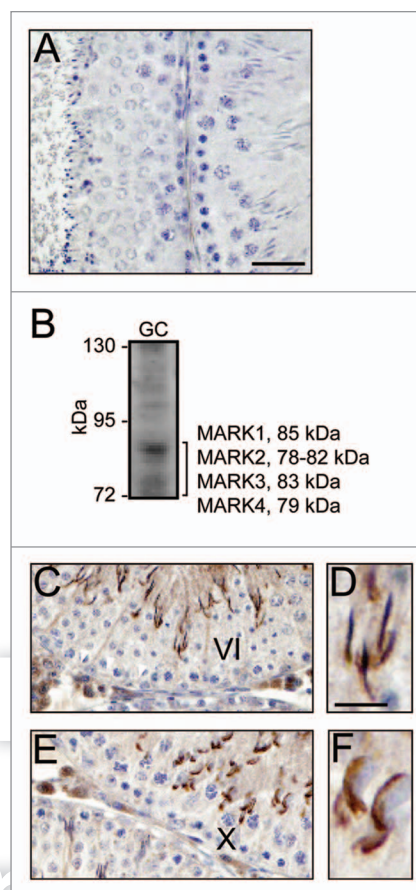
MARK4 in maintaining the integrity of the apical ES via its effects in stabilizing the tubulin cytoskeleton, we sought to use the adjuvin model to examine changes in the restrictive spatiotemporal expression of MARK4 at the apical ES during adjuvin-induced loss of cell polarity and premature release of spermatid from the seminiferous epithelium, mimicking spermiation.<sup>20,33,34</sup> It was noted that the adjuvin-induced disruption of spermatid polarity and spermatid loss from the seminiferous epithelium was found to associate with a significant decline in the steady-state level of MARK4 (Fig. 6A and B). There was a considerable “shift,” to be followed by a considerable loss of expression of MARK4 at the apical ES, mimicking the subtle decline and disappearance of MARK4 at the apical ES during the release of sperm at spermiation (Fig. 6C vs. 2). For instance, in both mis-oriented spermatids in which cell polarity was disrupted with their heads no longer pointing toward the basement membrane *and* prematurely departing elongating/elongated spermatids, MARK4 was found to diminish considerably, virtually disappearing from the apical ES (Fig. 6C), effectively mimicking elongated spermatids that undergo spermiation at late stage VIII of the epithelial cycle (Fig. 6C vs. 2). These findings thus support the notion that MARK4 is relevant to maintain apical ES stability via its effects in stabilizing tubulin cytoskeleton.

## Discussion

In the rat testis, there is a functional axis known as the “apical ES-BTB-hemidesmosome axis” which coordinates cellular events that take place at the opposite ends of the seminiferous epithelium during spermatogenesis,<sup>16,26</sup> such as the degeneration of the apical ES to facilitate the release of sperm at spermiation and restructuring of the BTB to facilitate the transit of preleptotene spermatocytes at the immunological barrier that occur simultaneously at stage VIII of the epithelial cycle. Biologically active fragments of laminin chains, such as domain IV of the laminin  $\gamma$ 3 and  $\beta$ 3 chains, that were released at the apical ES, likely via the action of MMP-2 (matrix metalloprotease-2) that was highly expressed at the apical ES prior to spermiation<sup>35</sup> on the  $\alpha$ 6 $\beta$ 1-integrin/laminin- $\alpha$ 3 $\beta$ 3 $\gamma$ 3 adhesion protein complex,<sup>36-39</sup> were found to perturb the Sertoli cell TJ-permeability barrier function at the BTB,<sup>26</sup> illustrating the presence of a functional axis between the apical ES and the BTB. Furthermore, these biologically active laminin fragments were also found to perturb the hemidesmosome function by reducing the expression of  $\beta$ 1-integrin at the site.<sup>26</sup> More important, a knockdown of  $\beta$ 1-integrin at the hemidesmosome by RNAi was found to perturb the BTB function, illustrating that a functional loop exists between the BTB and the hemidesmosome.<sup>26</sup> Since

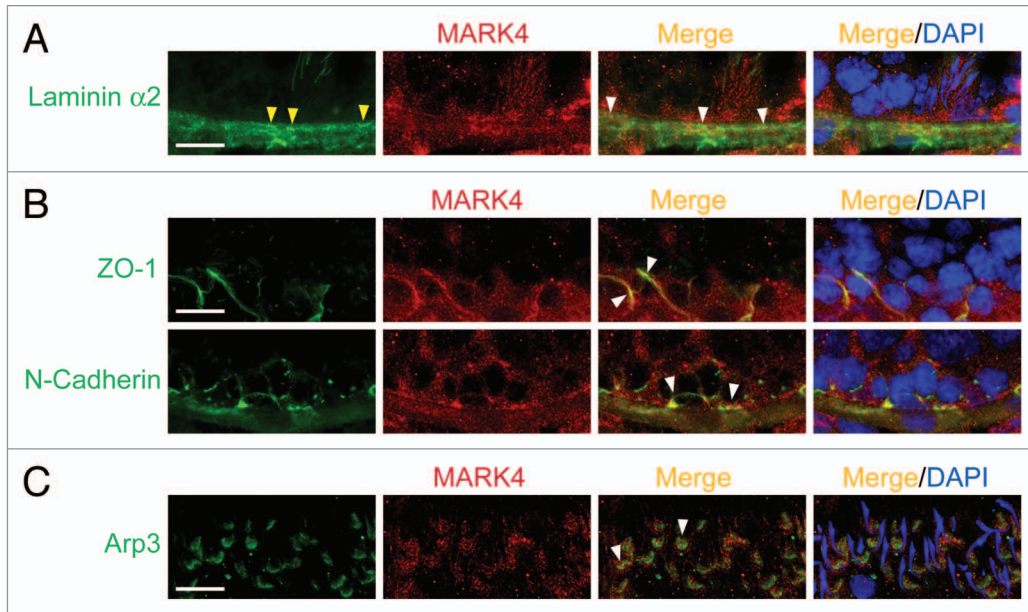
other studies have shown that a disruption of the basement membrane function<sup>40,41</sup> could perturb the integrity of the seminiferous epithelium, and the use of anti-collagen antibody [note: collagen  $\alpha 3$  (IV) is a major component of the basement membrane] was found to perturb the Sertoli cell BTB function,<sup>42</sup> illustrating a regulatory axis indeed exists between components of the basement membrane and the BTB. These findings coupled with the fact that hemidesmosomes (a cell-extracellular matrix anchoring junction type) are an integrated component of the basement membrane,<sup>16,43,44</sup> and that basement membrane is a modified form of extracellular matrix in the testis,<sup>45</sup> we thought it pertinent to rename the axis as the “apical ES-BTB-hemidesmosome/basement membrane” functional axis. Recent findings in the field, such as the use of the phthalate-induced Sertoli cell injury model, have supported the presence of this axis that coordinates and regulates cellular events that take place across the seminiferous epithelium during spermatogenesis.<sup>46–48</sup> While the identification of this axis is crucial to understand the intriguing regulation of cellular events across the axis, the major players along these axis that coordinate these cellular events, such as spermiogenesis and BTB restructuring, remain unknown.

The ES, both the apical ES at the Sertoli-spermatid (step 8–19 in the rat testis), and the basal ES at the Sertoli-Sertoli cell interface at the BTB, is morphologically similar, in which extensive network of bundles of actin filaments that lie perpendicular to the plasma membrane, and sandwiched in-between the cisternae of the endoplasmic reticulum and the apposing plasma membranes of: (1) Sertoli-spermatid interface at the apical ES or (2) Sertoli-Sertoli interface at the basal ES.<sup>16,21</sup> This network of filament bundles, however, is limited only to the Sertoli cell. These actin filament bundles also confer to the ES its unusual adhesive strength.<sup>22,23</sup> However, the ES must undergo restructuring to facilitate the timely movement of developing spermatids (i.e., step 8–19 in the rat testis) during spermiogenesis, as well as BTB restructuring during the epithelial cycle. This spermatid movement is possible only in the presence of the tubulin-based microtubule network that is found adjacent to the apical and basal ES,<sup>21,49</sup> which serves as the “tracks” for spermatid movement during spermiogenesis.<sup>21,24</sup> Earlier studies have demonstrated that Eps8 (epidermal growth factor receptor pathway substrate 8),<sup>50</sup> Arp3 (actin-related protein 3),<sup>51</sup> and drebrin E<sup>52</sup> are the three actin regulatory and actin binding proteins that elicit changes in the actin filament configuration at the apical ES to regulate spermatid movement. However, the regulation of the tubulin-based microtubule network in the testis remains unknown. Herein, we demonstrate for the first time the presence of a tubulin-stabilizing regulatory protein MARK4, which can be found in the testis, displaying highly restrictive spatiotemporal expression at the ES, in particular the apical ES, during spermiogenesis. However, at stage VII or early stage VIII when the apical ES begins to undergo degeneration to prepare for spermiation, MARK4 was intensively localized at the concave side of the apical ES where extensive endocytic vesicle-mediated protein trafficking takes place, creating an ultrastructure formerly known as the apical tubulobulbar complex (apical TBC),<sup>53–55</sup> suggesting that MARK4 is being used to facilitate protein trafficking events at the site,

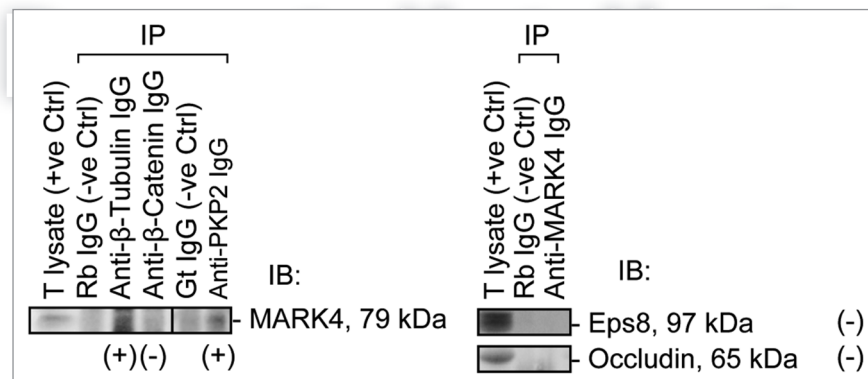


**Figure 3.** Localization of MARK1–4 in the seminiferous epithelium and its association with the apical and basal ES. The localization of MARK4 at the apical ES and the BTB in the seminiferous epithelium was further confirmed by using an anti-MARK1–4 antibody (see Table 1) with (A) illustrating the staining of the cross-section of adult rat testes with normal rabbit IgG to substitute the anti-MARK1–4 antibody to serve as a negative control. The specificity of this anti-MARK1–4 antibody (against the peptide NH<sub>2</sub>-LDTFC-COOH that was a consensus stretch of sequence shared by all MARKs proteins in humans) was shown by immunoblotting using lysates of germ cells (GC) (60  $\mu$ g protein) and a 7% SDS-polyacrylamide gel (B); and it is apparent that this anti-MARK1–4 antibody recognizes MARK4, 79 kDa, as the major MARK protein in the testis (see also Fig. 1A) (B). Similar to the localization of MARK4 in the testis (see Fig. 2), MARK1–4 was detected at the apical ES (C and D), surrounding the entire elongating spermatid head in a stage VI (C and D) and a stage X (E and F) tubule, as well as at the BTB (see “white” arrowheads in C). Bar in (A) = 40  $\mu$ m, which also applies to (C and E); bar in (D) = 15  $\mu$ m, which also applies to (F).

consistent with the reported functional role of MARKs in other epithelia.<sup>1,3</sup> However, the expression of MARK4 at the apical ES was found to reduce to a level almost non-detectable at late stage VIII when spermiation occurs, illustrating a complete loss of MARK4-mediated microtubule network stability at the site correlates with spermatid loss. This conclusion was supported by the study using the adjuvin model in which spermatids were depleted from the epithelium prematurely. Using this *in vivo* model, it was found that a loss of spermatid polarity, in which the heads of the elongating/elongated spermatids were pointing randomly instead of unidirectionally toward the basement membrane as of control



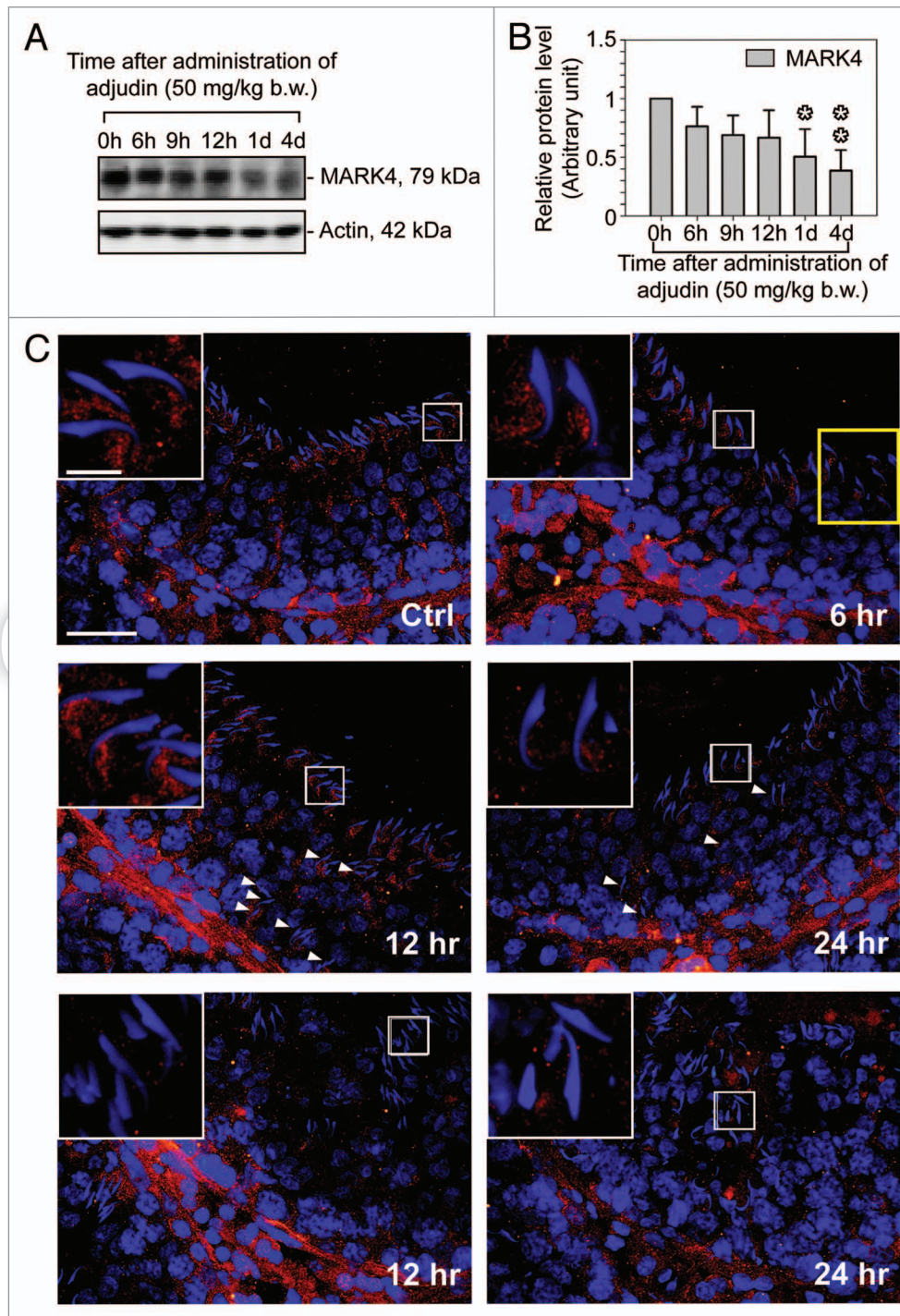
**Figure 4.** A study by dual-labeled immunofluorescence analysis to illustrate the localization of MARK4 at the hemidesmosome/basement membrane, the BTB, and the apical ES. Precise localization of MARK4 in the seminiferous epithelium was further investigated by its co-localization with corresponding markers of the hemidesmosome/basement membrane (e.g., laminin  $\alpha$ 2) (A), the BTB (e.g., ZO-1, a TJ adaptor; N-cadherin, a basal ES protein) (B), and the apical ES (e.g., Arp3)(C). The general site where hemidesmosomes are found is annotated by “yellow” arrowheads, and selected merged signals are annotated by “white” arrowheads. Bar in the first micrograph in A, B or C = 40  $\mu$ m, which applies to remaining micrographs.



**Figure 5.** A study by co-immunoprecipitation (Co-IP) to identify the binding partners of MARK4 in adult rat testes. Using about 800  $\mu$ g protein of lysates from adult rat testes for each Co-IP reaction as described in Materials and Methods with the corresponding antibodies, MARK4 was found to structurally interact with  $\beta$ -tubulin and desmosomal armadillo protein plakophilin-2, but not with any actin-based adhesion proteins at the basal ES (e.g.,  $\beta$ -catenin), apical ES (e.g., Eps 8) and TJ (e.g., occludin, Eps8). T lysate, testis lysate; IP, immunoprecipitation; IB, immunoblotting; Rb, rabbit; Gt, goat; Ctrl, control; IgG, immunoglobulin G; (+), positive protein-protein interaction; (-), negative protein-protein interaction.

rat testes, was associated with a subtle loss of MARK4 at the apical ES *and* the premature release of elongating/elongated spermatids from the epithelium. These findings thus demonstrate unequivocally that the tubulin-based cytoskeleton regulated by MARK4 is working in concert with other polarity proteins, such as PAR3 (partitioning-defective protein 3), PAR6, Cdc42 and 14-3-3 (also known as PAR5), associated with the actin-based cytoskeleton to regulate spermatid polarity and adhesion along the apical ES-BTB-hemidesmosome/basement membrane axis. Furthermore, the demonstration that MARK4 structurally interacts with desmosomal adaptor plakophilin-2 illustrates that this

protein may also play a role in regulating desmosome at the BTB. In summary, these findings reported herein thus provide the basis for designing functional experiments to understand and resolve unanswered questions concerning the role of tubulin cytoskeleton in this axis. For instance, does the restrictive spatiotemporal expression of MARK4 in the seminiferous epithelium serve as the molecular “switch” that turns “on” and “off” regarding the stability of the tubulin cytoskeleton to induce changes in adhesion at the apical ES and basal ES during the epithelial cycle of spermatogenesis?



**Figure 6.** For figure legend, see page 124.

## Materials and Methods

**Animals and antibodies.** The use of Sprague-Dawley rats for studies reported herein was approved by the Rockefeller University Institutional Animal Care and Use Committee (IACUC) with Protocol Numbers 09-016 and 12-506. Rats were housed at the Rockefeller University Comparative Bioscience Center (CBC) with free access to standard rat chow and water ad libitum at

$22 \pm 1^\circ\text{C}$  with a light:dark cycle of 12 h:12 h, according to the applicable portions of the Animal Welfare Act and the guidelines in the Department of Health and Human Services publication “Guide for the Care and Use of Laboratory Animals”.

**The adjuvins animal model.** Adult rats (~300 g b.w.) were treated with a single dose of adjuvins, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide, a potential male contraceptive known to induce germ cell loss, most notably elongating

**Figure 6 (See previous page).** A study using the adjuvins model to illustrate MARK4 maintains the tubulin cytoskeleton stability at the apical ES. Adult rats (~300 g b.w.) were treated with a single dose of adjuvins (50 mg/kg b.w.) at time 0 h (hour), which is known to induce germ cell loss,<sup>34</sup> initially elongating/elongated spermatids, to be following by round spermatids and spermatocytes. Changes in the steady-state level of MARK4 was quantified by immunoblotting shown in (A) using  $\beta$ -actin as a protein loading control. (B) This histogram summarizes the composite data shown in (A) with each bar = mean  $\pm$  SD of  $n = 3$  rats. Changes in the localization of MARK4 in the seminiferous epithelium was also assessed by immunofluorescence microscopy as shown in (C). In the control (Ctrl) testis, such as an early stage VIII tubule, MARK4 localized at the concave side of the elongating spermatids began to move away from this site, similar to data shown in Figure 2 to prepare the release of sperm at spermiation during the epithelial cycle. However, in rats treated with adjuvins by 6 h, while MARK4 was still found at the concave side of the elongating spermatid head (see inset), MARK4 diminished considerably in the apical ES in most elongating spermatids (see spermatids encircled by the "yellow" box). By 12- and 24-h after adjuvins treatment (middle part), many elongating spermatids remained "trapped" in the seminiferous epithelium, many of which lost their polarity and became mis-oriented with their heads no longer pointing toward the basement membrane and the level of MARK4 was also considerably diminished or even absence (see "white" arrowheads). However, in many tubules at 12- and 24-h after adjuvins treatment (bottom part), mis-oriented elongating spermatids, round spermatids and even spermatocytes were depleting from the epithelium and the staining of MARK4 vanished surrounding the heads of these spermatids, noticeably different from the control rat testis (see Ctrl micrograph). Selected area in each of these micrographs was encircled by a "white" box which was magnified and shown in an inset. In Ctrl, bar = 50  $\mu$ m, which applies to all other micrographs; bar in inset in Ctrl = 10  $\mu$ m, which also applies to all other insets.

spermatids by disrupting the apical ES,<sup>20,34,56,57</sup> at 50 mg/kg b.w. by gavage (working dilution of adjuvins at 20 mg/ml, suspended in 0.05% methylcellulose/99.5% MilliQ water).<sup>58</sup> Thereafter, groups of rats with  $n = 3-6$  animals per time point, including both treatment and control groups, were euthanized by CO<sub>2</sub> asphyxiation. Testes were removed, and one testis from each rat was fixed in Bouin's fixative to be embedded in paraffin wax, and the other testis from the same rat was snap-frozen in liquid nitrogen for at least 5 min, before it was stored at  $-80^{\circ}\text{C}$  until used for either preparation of lysates for immunoblotting<sup>59,60</sup> or to obtain frozen sections (7- $\mu$ m in thickness at  $-22^{\circ}\text{C}$  in a cryostat) for immunohistochemistry. This animal model was selected so that changes in the expression and/or localization of MARK4 (Note: MARK4 is a regulator of microtubule dynamics) during adjuvins-induced spermatid loss from the seminiferous epithelium could be correlated with apical ES disruption (Note: ES is an actin-based testis-specific adherens junction type). This thus assesses the functional relationship between actin filament bundles and the microtubule filaments at the apical ES since the movement of spermatids during spermiogenesis and the release of spermatid at spermiation<sup>16,61</sup> involves the apical ES, and this is intimately related to microtubules besides actin filaments,<sup>21,24</sup> yet there is no report in the literature to address the functional relationship between these two cytoskeletons in the testis.

**Primary testicular cell cultures.** Sertoli cells were isolated from 20-d-old rat testes.<sup>62</sup> These cells were differentiated, ceased to divide, and mimicked Sertoli cells isolated from adult rat testes morphologically, physiologically and functionally.<sup>63,64</sup> Sertoli cells were cultured in serum-free F12/DMEM (Ham's F12 nutrient mixture/Dulbecco's modified Eagle's medium) supplemented with growth factors<sup>62</sup> at  $35^{\circ}\text{C}$  in a humidified CO<sub>2</sub> incubator with 95% air/95% CO<sub>2</sub> (v/v)<sup>62</sup> for 4 d to allow the assembly of a functional TJ-permeability barrier that mimicked the Sertoli cell BTB in vivo.<sup>65,66</sup> Thereafter, these cultures were terminated and lysates were obtained,<sup>59</sup> Germ cells were isolated from adult rat testes using a mechanical procedure,<sup>67</sup> and cultured in F12/DMEM supplemented with 2 mM sodium pyruvate and 6 mM sodium DL-lactate for ~16 h. The viability of these germ cells was at least 96% as judged by erythrosine red dye exclusion test<sup>68</sup> when these cultures were terminated for lysate preparation.

**Immunofluorescence microscopy.** Immunofluorescence microscopy and dual-labeled immunofluorescence analysis was performed<sup>51,59</sup> using frozen sections (7  $\mu$ m in thickness) of testes obtained in a cryostat at  $-22^{\circ}\text{C}$ . To avoid inter-experimental variations, all sections of testes from rats within an experimental group including both treated vs. control rats were processed in a single experimental session by mounting all sections on a minimal number of poly-L-lysine-coated microscope slides (Polysciences). Sections were fixed in paraformaldehyde (w/v), permeabilized in 0.1% Triton X-100 (v/v), blocked in 1% BSA (w/v), followed by an overnight incubation with anti-MARK4 antibody (Table 1), and then an Alexa Fluor-conjugated secondary antibody (Invitrogen; Alexa Fluor 555). Fluorescence images were acquired using a Nikon Eclipse 90i Fluorescence Microscope System and Nikon NIS Elements Advanced Research software package and converted to JPEG format. All images were subsequently assembled using Adobe InDesign in Adobe Creative Suite 3.0.

**Immunohistochemistry (IHC).** IHC was performed,<sup>65</sup> with minor modifications. In brief, testes were fixed in Bouin's fixative (Polysciences) for 24 h, dehydrated using increasing concentrations of ethanol, cleared with xylene, embedded in paraffin and sectioned. After deparaffinization in xylene and rehydration, antigen retrieval was performed in 10 mM citrate buffer, pH 6.0 at  $22^{\circ}\text{C}$ , for 10 min in a microwave. Other procedures, including incubation with the anti-MARK1-4 antibody (see Table 1), were performed.<sup>65</sup> Thereafter, sections were incubated with biotinylated goat anti-rabbit IgG at 1:400 dilution, followed by VECTASTAIN Elite ABC (Avidin: Biotinylated enzyme Complex) reagent (Vector Labs). Color development was performed using DAB (3,3'-diaminobenzidine). Micrographs were obtained using the Nikon Eclipse 90i microscope and Nikon NIS Elements Advanced Research Imaging Software package in JPEG format. All micrographs were subsequently analyzed in PhotoShop using Adobe Creative Suite 5.0.

**Immunoblotting.** Lysates from Sertoli and germ cell cultures, and testes were obtained using lysis buffer [10 mM Tris, 0.15 M NaCl, 1% NP40 (Tergitol-type nonyl phenoxypolyethoxyethanol, v/v) and 10% glycerol (v/v), pH 7.4 at  $22^{\circ}\text{C}$ ]. Protease and phosphatase inhibitor cocktails (Sigma-Aldrich) were added to the lysis buffer immediately prior to its use at 1:100 dilution according to instructions from the manufacturer. Protein concentration



was estimated by DC Protein Assay (Bio-Rad Labs). SDS-PAGE and immunoblotting were performed<sup>59</sup> to visualize MARK1–4 or MARK4 in blots. Enhanced chemiluminescence was performed using a luminol/p-coumaric acid-based home-made kit.<sup>69</sup> Chemiluminescence images were obtained using a Fujifilm LAS-4000 mini imaging system, and images were acquired in TIFF format using Multi Gauge (Version 3.1) in Science Lab 2005 software package (Fujifilm Corp.).

**Co-immunoprecipitation (Co-IP).** To identify binding partners of MARK4 in the adult testis, co-immunoprecipitation was performed.<sup>59,70</sup> In brief, rat testis lysates (~800 µg protein in each reaction tube) were first pre-cleared with the corresponding animal serum (or IgG) as described,<sup>59</sup> to eliminate non-specific antibody-antigen interactions. Thereafter, the immunoprecipitation antibody was added, samples were incubated overnight at room temperature and the immunocomplexes were precipitated

using Protein A/G Plus (Santa Cruz). Binding partners were then identified by immunoblotting using corresponding antibodies (see Table 1).

**Statistical analysis.** Each experiment was repeated at least three times with n = 3–6 rats or using different batches of testicular cells. Statistical analysis was performed by one-way ANOVA and post hoc Dunnett's test using GB-STAT (Version 7.0, Dynamic Microsystems).

#### Acknowledgements

This work was supported by grants from the National Institutes of Health (NICHD R01 HD056034 to C.Y.C.; U54 HD029990 Project 5 to C.Y.C.); and Government of India, Department of Biotechnology, BT/BI/03/015/2002 to P.P.M., Department of Information Technology, DIT/R&D/BIO/15(9)/2007 to P.P.M.,

and University Grants Commission to P.J. for University Research Fellowship..

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