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INVITED REVIEW

Sperm Biology

Plastins regulate ectoplasmic specialization via its actin bundling activity on microfilaments in the rat testis

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Plastins are a family of actin binding proteins (ABPs) known to cross-link actin microfilaments in mammalian cells, creating actin microfilament bundles necessary to confer cell polarity and cell shape. Plastins also support cell movement in response to changes in environment, involved in cell/tissue growth and development. They also confer plasticity to cells and tissues in response to infection or other pathological conditions (e.g., inflammation). In the testis, the cell-cell anchoring junction unique to the testis that is found at the Sertoli cell-cell interface at the blood-testis barrier (BTB) and at the Sertoli-spermatid (e.g., 8–19 spermatids in the rat testis) is the basal and the apical ectoplasmic specialization (ES), respectively. The ES is an F-actin-rich anchoring junction constituted most notably by actin microfilament bundles. A recent report using RNAi that specifically knocks down plastin 3 has yielded some insightful information regarding the mechanism by which plastin 3 regulates the status of actin microfilament bundles at the ES via its intrinsic actin filament bundling activity. Herein, we provide a brief review on the role of plastins in the testis in light of this report, which together with recent findings in the field, we propose a likely model by which plastins regulate ES function during the epithelial cycle of spermatogenesis via their intrinsic activity on actin microfilament organization in the rat testis.

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INTRODUCTION

Plastins are a family of actin binding proteins (ABPs) having the intrinsic activity of conferring actin microfilament bundling, promoting long stretches of microfilaments with the same polarity to be bundled.^{1–5} This actin bundling capability of a single plastin polypeptide is mediated by the two tandem pairs of CH (calponin homology) domain in its polypeptide sequence creating two ~27 kDa actin binding domains (ABD1 and ABD2) (**Figure 1**) that are being used to create actin microfilament bundles, such as those found in the ectoplasmic specialization (ES) at the Sertoli-Sertoli and Sertoli-spermatid interface in the mammalian testis, which are also essential to confer Sertoli cell and spermatid adhesion, polarity and spermatid transport.^{6–8} The N-terminal region of plastin contains a calcium ion-binding domain (CaBD) composed of two EF hand motifs⁹ (**Figure 1**). The first member of the plastin ABP family found in vertebrates is plastin 1 (also known as I-plastin or intestine plastin) (**Table 1** and **Figure 1**). It was first identified in 1979 in microvilli from intestinal brush border of chicken as a 68 kDa polypeptide, shown to be involved in the organization of actin microfilament bundles to constitute the microvillus core filaments,¹⁰ and subsequently shown to be expressed by cells of the colon, and kidney, besides small intestine in humans.¹¹ It was also called fimbrin as it was found to associate with other cell

surface structures such as focal adhesions, microspikes and membrane ruffles, besides microvilli, in fibroblasts and mammary cells in studies *in vitro*.¹² Fimbrin was subsequently shown to bind and cross-link actin microfilaments,^{13,14} thereby creating polarized actin microfilament bundles in mammalian cells to support cell polarity, cell adhesion, locomotion, and other cellular events. At about the same time, another 68 kDa protein called plastin 2 (also known as L-plastin) that confers actin microfilament bundles was found to be highly expressed in lymphocytes,¹⁵ leukocytes¹⁶ (i.e., hematopoietic cells), and also cancer cells such as transformed human fibroblasts,^{17,18} also known as lymphocyte cytosolic protein 1 (LCP1) (**Table 1** and **Figure 1**). Plastin 2 is known to be involved in T-cell motility and activation,¹ and plastin 2 deficient neutrophils fail to kill bacterial pathogens¹⁹ since these cells lack the ability to quickly rearrange their actin cytoskeleton in response to the pathogens. Plastin 3 (also known as T-plastin), also a 68 kDa monomeric polypeptide, is found in cells derived from solid tissues such as neurons, auditory hair cells, melanocytes, and osteoblasts^{20–23} (**Table 1** and **Figure 1**). Defects of plastin 3 in humans due to mutation are known to associate with osteoporosis, leading to bone fracture.²⁴ This is possibly due to defects in the organization of actin filament bundles in bone cells, perturbing the necessary conversion of mechanical signals to biochemical signals in osteoblasts

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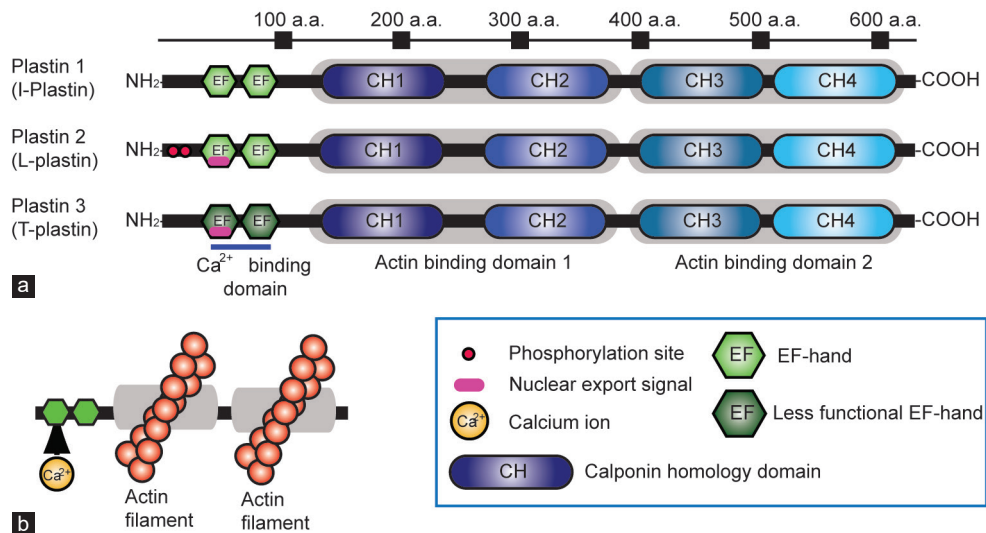


Figure 1: Schematic illustration of the various common functional domains in members of the plastin protein family. (a) Plastin, such as plastin 2 (L-plastin) is known to have two putative phosphorylation sites (e.g., Ser-5 and -7) near its N-terminus. The phosphorylation of these sites is known to activate plastin 2 to unleash its intrinsic actin bundling activity, but similar phosphorylation site(s) in plastin 1 and 3 remains to be identified. It is followed by the two EF-hand, a helix-loop-helix structural domain (or motif), found in a large family of calcium-binding proteins, along with the nuclear export signal sequence. There are also two actin binding domains (ABDs) of ABD1 and ABD2, each is comprised of two in tandem calponin homology (CH) domains. (b) The binding of two actin microfilaments in each molecule of plastin thus induces actin microfilament bundling such as those found at the ES.

Table 1: Function of plastins

Protein/gene	Tissue expression	Binding partner	Mutation or deletion and human disease**	KO or KD on fertility or spermatogenesis	Phenotype(s) of KO or KD in rodents
Plastin 1 (I-plastin also known as fimbrin), encoded by the <i>PLS1</i> gene mapped to chromosomes 3 ¹¹ Mr, 68 kDa	Small intestine, ⁵¹ stereocilia ⁵²	Actin, ¹² keratin ²⁸	Not known	Plastin 1 deficient mice are fertile ²⁸	The microvilli of plastin 1 deficient mice are shorter and without rootlets, brush borders are highly fragile, and KO mice are sensitive to DSS-induced colitis; ²⁸ and hearing was impaired in adult plastin 1 KO mice ⁵²
Plastin 2 (L-plastin also known as L-fimbrin and LCP1), encoded by the <i>LCP1</i> gene mapped to chromosomes 13 ⁵¹ Mr, 68 kDa	Hematopoietic cells ⁵¹	Actin, ⁵³ vimentin, ⁵⁴ Iba1, ⁵⁵ Rab5 ⁵⁶	Mutation of plastin 2 (LCP1) is likely a genetic marker for preclinical diagnosis of retinoblastoma based on a case report ⁵⁷	Knockout of plastin 2 did not affect embryonic or neonatal development ^{19,58}	Thymocytes failed to exit the thymus normally, leading to increased mature spleen thymocytes in plastin 2 KO mice, germinal center formation and T-dependent antibody generation are reduced in plastin 2 KO mice ^{19,58-60}
Plastin 3 (T-plastin or T-fimbrin), encoded by the <i>PLS3</i> gene mapped to chromosomes X ⁵¹ Mr, 68 kDa	Epithelial and mesenchymal cells, solid tissue except jejunum and ileum of the small intestine ⁵¹	Actin, ⁴³ vimentin, ²⁷ Rab5 ⁵⁶	Plastin 3 mutation induces osteoporosis, ^{24,61} PLS3 is important for axonogenesis in humans; ²³ PLS3 acts as a protective modifier of SMA in humans that have homozygous deletion of the SMN1 gene ²³	Plastin 3 KD <i>in vivo</i> and <i>in vitro</i> perturbs the organization of actin filaments in Sertoli cells at the ES in rats ²⁷	Zebrafish with plastin 3 KD had severe dysplasia of craniofacial skeletal elements, muscle in plastin 3 KD larvae is also deformed; ²⁴ overexpression of plastin 3 in zebrafish rescued the axon length and outgrowth defects caused by the loss of SMN protein, the SMA-determining gene product; ²³ KD of plastin 3 in adult rat testes induces failure in spermatid and phagosome transport ²⁷

**Mutation or deletion of any plastin gene that may impede male fertility in humans in particular the status of spermatogenesis is not known since there is no report in the literature regarding the role of human plastin proteins in human testes. DSS: dextran sulfate sodium; Iba1: ionized calcium binding adaptor molecule 1; kDa: kilodalton; KD: knockdown; KO: knockout; LCP1: lymphocyte cytosolic protein 1; Mr: molecular weight; *PLS*: *plastin* gene; SMN: survival motor neuron; SMA: spinal muscular atrophy; SMN1: survival motor neuron 1

and osteoclasts rapidly,²⁵ leading to osteoporosis.²⁶ Interestingly, a study of homozygous deletion of the survival motor neuron 1 (SMN1) gene that causes spinal muscular atrophy (SMA) – a genetic disorder leading to early childhood lethality – using both a mouse and a zebrafish model has shown that overexpression of plastin 3 can repair neuronal defects associated with the SMA disorders, such as the axon length and outgrowth defects, illustrating plastin 3 is a protective modifier of SMA.²³ Studies have shown that there is ~75% sequence similarity between all three plastins found in mouse and human^{3,22} (Table 2), and the similarity of plastin 1, 2 and 3 between mouse and human proteins

are of 94%, 97% and 99%, respectively, based on the homology analysis tools in BLAST (Basic Local Alignment Search Tool) from National Center for Biotechnology Information using corresponding protein sequence data of plastins in GenBank (Table 2). Interestingly, Sertoli cells were found to express all three members of the plastin ABP protein family: plastin 1, 2 and 3, whereas germ cells expressed only plastin 1 and 2,²⁷ illustrating nature has installed multiple actin bundling proteins to protect the integrity of actin microfilament bundles at the ectoplasmic specialization (ES) to support cell adhesion function and spermiogenesis – the two essential functions of the ES. Thus, it is not

Table 2: Amino acid homology between plastins in humans and mice

Amino acid homology (%)	Human plastin		
	Plastin 1: NP_001165783.1	Plastin 2: NP_002289.2	Plastin 3: EAX02614.1
Mouse plastin			
Plastin 1: NP_001028382.1	94	74	75
Plastin 2: NP_001234913	75	97	80
Plastin 3: NP_001159926.1	75	80	99

surprising that mutation or deletion of either plastin 1,²⁸ 2¹⁹ or 3²⁴ in humans or mice did not seem to affect fertility since it is likely that the lost of one plastin can be superseded by the other two members of the family in the testis. Thus, it will be of interest to examine if a triple knockdown of all three plastins would affect fertility by impeding spermatogenesis. However, a recent report has shown that the knockdown (KD) of plastin 3 in Sertoli cells by RNAi indeed perturbs the organization of actin microfilaments in Sertoli cells and also the ES in the testis in studies of *in vitro* and *in vivo*, illustrating the functional significance of plastin 3 in spermatogenesis. Herein, we briefly describe these findings and critically evaluate the role of plastin 3 in the testis in light of findings in the field.

PLASTINS AND THE TESTIS

In the mammalian testis, when the cross-section of a seminiferous tubule is examined microscopically, the seminiferous epithelium is notably divided into the adluminal (apical) and the basal compartment by the blood-testis barrier (BTB). Under electron microscope, the most prominent cell junction detected in the seminiferous epithelium of the adult rat testis is the ectoplasmic specialization (ES) first named in 1977^{29,30} at the Sertoli cell-cell interface at the BTB and at the Sertoli-spermatid interface, designated basal and apical ES, respectively, referring on their relative location in the seminiferous epithelium. While the morphological features and the possible physiological function of the ES are known for several decades, the mechanisms by which the ES regulates Sertoli and spermatid polarity, adhesion, and spermatid transport are beginning to emerge until recent years.^{6,7,31–33} The ES, unlike other cell-cell anchoring junctions that also use F-actin for their attachment, is a testis-specific actin-rich anchoring junction. The apical ES anchors spermatids (steps 8–19) onto the Sertoli cell in the seminiferous epithelium, it is composed of only one array of actin filament bundles restricted to the Sertoli cell at the Sertoli cell-spermatid interface, and no corresponding ultrastructure is found in the spermatid (**Figure 2**). Furthermore, once apical ES appears in step 8 spermatids, it replaces all other junctions (e.g., gap junction and desmosome) and is the only anchoring device remaining throughout spermiogenesis. On the other hand, the basal ES, is restricted to the Sertoli cell-cell interface at the BTB, is formed by two arrays of actin filament bundles, each of which is found in one of the two adjacent Sertoli cells (**Figure 3**). Unlike apical ES, basal ES coexists with TJ and gap junction, which together with the intermediate filament-based desmosome create the BTB. At the ES, actin microfilaments that lie perpendicular to the Sertoli cell plasma membrane are sandwiched in-between cisternae of endoplasmic reticulum (ER) and the apposing Sertoli cell-spermatid (apical ES) (**Figure 2**) or Sertoli cell-cell (basal ES) plasma membrane (**Figure 3**). Due to the intrinsic actin bundling activity of plastins (**Figure 1**), it is conceivable that these ABPs are crucial for the assembly and regulation of actin microfilaments at the

ES during the epithelial cycle. Surprisingly, it is almost four decades since the discovery of the first plastin namely plastin 1 (fimbrin), there are few published reports in the literature that examined the functional significance of plastins in the ES except an earlier study in 1989, which was almost 10 years after the initial discovery of plasmin 1 (fimbrin), that a fimbrin-like 83 kDa protein was identified in the SDS-extract of the ES from rodent testes by immunoblotting.³⁴ In this study, fimbrin and vinculin were detected in the SDS-extract of ES and suggested that fimbrin might be used to cross-link actin microfilaments at the ES to confer a unique testis anchoring junction.³⁴ Since then, no functional study can be found in the literature. In order to better understand the regulation of actin microfilaments at the ES, we recently report findings based on the use of plastin 3-specific siRNA duplexes by RNAi for its knockdown in Sertoli cells cultured *in vitro* with an established functional tight junction (TJ)-permeability barrier that mimics the Sertoli cell BTB *in vivo*, as well as its knockdown in the testis *in vivo*.²⁷ Sertoli cell express all three plastins with plastin 3 being the predominant form, whereas total germ cells express plastin 1 and 2, but not plastin 3.²⁷ Plastin 3 also co-localizes with actin microfilaments in Sertoli cells.²⁷ The expression of plastin 3 in the seminiferous epithelium displays restrictive spatiotemporal pattern.²⁷ For instance, plastin 3 is expressed at the basal ES of the BTB in virtually all stages of the epithelial cycle but most prominently in stages V–VII tubules and considerably diminished in stage VIII tubules when the BTB undergoes remodeling/restructuring, perhaps being used to accommodate the transport of preleptotene spermatocytes across the barrier by modifying the organization of actin microfilament bundles at the basal ES.²⁷ At the apical ES, plastin 3 is expressed almost exclusively in stage VII tubules, localized restrictively to the tip of spermatid heads, co-localized with nectin-3,²⁷ which is a spermatid-specific apical ES protein that forms an adhesion protein complex with nectin-2 in Sertoli cells.³⁵ Following a knockdown of plastin 3 by RNAi in Sertoli cells cultured with an established functional TJ barrier, it was noted that actin microfilaments failed to align as bundles across the Sertoli cell cytosol, instead, they were mis-aligned across cell cytosol and some microfilaments were truncated with shorter stretches of filaments, but the vimentin-based intermediate filaments were unaffected in these cells.²⁷ This, in turn, failed to support proper localization of actin-based basal ES protein complexes, such as N-cadherin- β -catenin, since these proteins were found to be grossly internalized, re-distributed from near the cell surface to cell cytosol, thereby perturbing the Sertoli cell TJ-permeability barrier function.²⁷ Surprisingly, the localization of TJ-based protein complex, such as claudin-11/ZO-1, at the Sertoli cell surface was unaffected; this thus explains the partial maintenance of the Sertoli cell TJ barrier function following a mis-localization of basal ES proteins after plastin 3 knockdown.²⁷ These findings also suggest that other plastins found in the testis, such as plastin 1 and plastin 2 expressed by Sertoli cells, or other actin bundling proteins, such as ezrin,³⁶ palladin,³⁷ and Eps8³⁸ found in the testis may supersede the lost function of plastin 3. It is also noted that a knockdown of plastin 3 in Sertoli cells also impedes the proper localization of an actin barbed end capping and bundling protein Eps8, actin cross-linking and bundling protein palladin, as well as the branched actin polymerization protein Arp3.²⁷ The mis-localization of these other actin binding and regulatory proteins could also be the result of changes in the organization of actin microfilaments across the Sertoli cell following plastin 3 knockdown, so that they could no longer localize properly as found in normal Sertoli cells. Based on these findings, we now propose two hypothetical models by which plastin 3 regulates actin microfilament organization at the apical (**Figure 2**) versus basal (**Figure 3**) ES, facilitating endocytic

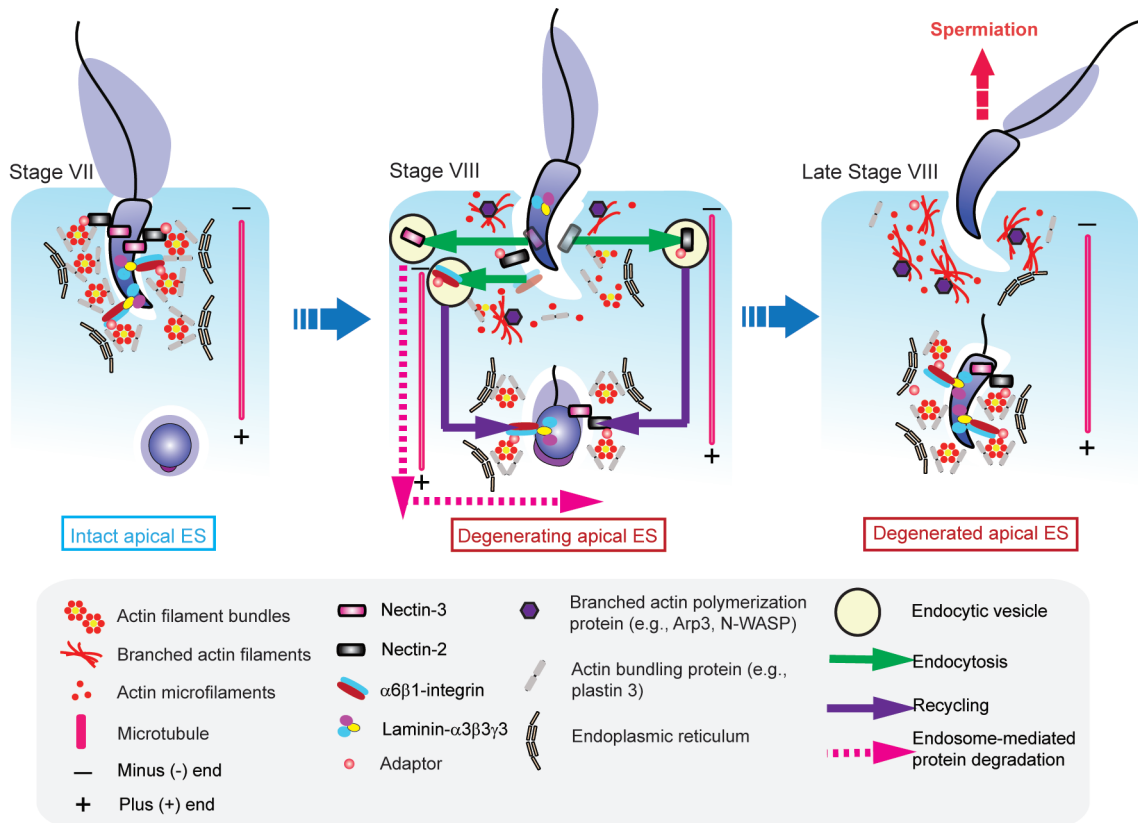


Figure 2: A hypothetical model illustrating the role of plastins in the remodeling of the apical ES during the epithelial cycle of spermatogenesis. The left panel is a schematic drawing of a stage VII tubule illustrating the apical ES is intact with functional adhesion protein complexes, such as nectin-afadin and integrin-laminin, utilizing F-actin as the attachment site to confer spermatid adhesion onto the Sertoli cell in the seminiferous epithelium in the adluminal (apical) compartment. The actin filament bundles are maintained by the actin bundling proteins such as plastin 3. From late stage VII through early stage VIII (middle panel), actin microfilaments are becoming un-bundled, via the combined action of an up-regulation of the barbed end branched actin nucleation protein Arp2/3 complex/N-WASP that generate branched actin filaments, and a down-regulation of actin cross-linking and bundling protein plastin 3. This thus converts actin microfilaments from a bundled to a un-bundled/branched configuration, facilitating endocytic vesicle-mediated protein trafficking events of endocytosis, and recycling to assemble “new” apical ES derive from step 8 spermatids that arise in stage VIII tubules versus endosome-mediated protein degradation. These endocytic vesicle-mediated trafficking events are also facilitated by the presence of polarized microtubules (MTs) that serve as the track for the intracellular transport of these vesicles. In late stage VIII (right panel), the extensive degeneration of apical ES facilitates the release of fully developed spermatids (i.e., spermatozoa) at spermiation. At the same time (see right panel), elongating spermatids also develop progressively with intact apical ES.

vesicle-mediated protein trafficking. As such, “old” apical as well as “old” basal ES/BTB proteins can be endocytosed and recycled for the assembly of “new” apical and BTB proteins, respectively. These changes as depicted in **Figure 2** thus accommodate the release of fully developed elongated spermatids (i.e., spermatozoa) at spermiation, and the transport of preleptotene spermatocytes across the barrier at stage VIII as shown in **Figure 3**. It is conceivable that plastin 3 is not working alone, instead, it is working in concert with a number of structural and regulatory proteins that effectively organize actin microfilaments and also MT-based cytoskeleton for the transport of spermatids versus preleptotene spermatocytes (**Figures 2 and 3**).

PLASTIN 3 AND SPERMATOGENESIS

When plastin 3 was silenced in the testis *in vivo* by RNAi, thereby perturbing the organization of actin microfilaments at the ES, the most obvious phenotypes found in the seminiferous epithelium were a distinctive failure of: (i) spermatid transport and (ii) phagosome transport.²⁷ For instance, elongated spermatids (e.g., step 19 spermatids) were found to be embedded deep inside the epithelium in stage VIII tubules, and some step 19 spermatids were also detected near the basement membrane even in stage X tubules and were

present among step 10 spermatids,²⁷ illustrating a failure in spermatid transport at these stages. Interestingly, phagosomes derived from residual bodies engulfed by Sertoli cells that were found near the basement membrane in stage IX tubules in control testes³⁹ remained at the adluminal edge of the epithelium, in stage IX tubules after plastin 3 KD in the testis as of stage VIII tubules.²⁷ These findings thus confirm the concept that actin microfilament bundles at the ES maintained by plastins are being used to confer transport of spermatids and other organelles (e.g., phagosomes) during the epithelial cycle. The lack of significant impact on the status of spermatogenesis following specific KO of either plastin 1, 2 or 3 as noted in **Table 1** is likely that other plastins as well as other actin bundling proteins can supersede the loss of one of the plastin genes. This notion, in fact, is supported by observations on the actin microfilament organization in Sertoli cells following plastin 3 KD, since the silence of plastin 3 by ~70% using RNAi was found to induce extensive truncation of actin microfilaments in Sertoli cells, some branched actin microfilaments were notably detected, even though the localization of TJ-based adhesion protein complexes (e.g., claudin-11/ZO-1) remained relatively unaffected.²⁷ However, it is noted that mis-localization of basal ES proteins (e.g., N-cadherin/ β -catenin) was grossly affected

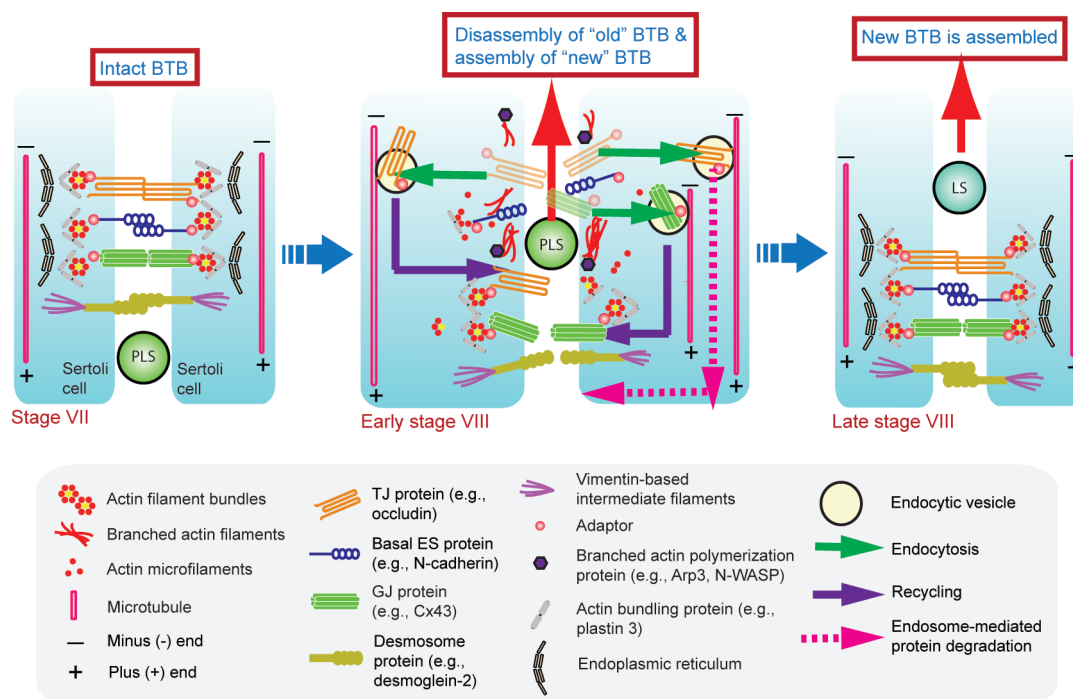


Figure 3: A hypothetical model illustrating the role of plastins in the remodeling of the basal ES at the BTB during the epithelial cycle of spermatogenesis to facilitate the transport of preleptotene spermatocytes across the immunological barrier. Preleptotene spermatocytes transformed from type B spermatogonia that are detected at stage VII of the epithelial cycle are being transported across the BTB as shown in this schematic drawing, involving extensive remodeling of actin microfilament bundles at the basal ES. The left panel depicts the schematic drawing of a cross-section of the seminiferous epithelium in a stage VII tubule in which an intact BTB is located above the preleptotene spermatocyte (PLS). The BTB is composed of F-actin-based TJ (e.g., occludin, JAM-A, CAR, ZO-1), basal ES (N-cadherin, β -catenin, nectin-2, afadin), and GJ (gap junction) (e.g., connexin 43, connexin 33) proteins, as well as intermediate filament-based desmosome proteins (e.g., desmoglein-2, desmocollin-2). In late to early stage VIII (see middle panel), actin microfilament bundles in the "old" BTB above the PLS are becoming unbundled, via the combined action of an up-regulation of the barbed end branched actin nucleation protein Arp2/3 complex/N-WASP that generate branched actin filaments, and a down-regulation of actin cross-linking and bundling protein plastin 3, perhaps involving other actin bundling proteins such as ezrin, palladin, and fascin 1. This thus converts actin microfilaments from a bundled to an unbundled/branched configuration, facilitating endocytic vesicle-mediated protein trafficking events of endocytosis, and recycling so that a "new" BTB can be assembled behind the PLS. Other unwanted internalized "old" BTB proteins can also be subjected to endosome-mediated degradation. This timely sequence of "old" BTB breaks down versus "new" BTB reassembly thus facilitates the transport of PLS across the barrier until a "new" BTB is established as shown in the right panel when PLS has transformed to leptotene spermatocyte (LS) in early stage IX of the epithelial cycle.

and perturbed.²⁷ In this context, it is of interest to note that while plastin 3 is important to confer actin bundles at the ES, it can also impede actin microfilament turnover because plastin is known to stabilize F-actin network in cells.^{40,41} Thus, plastin is likely working in concert with cofilin, an actin depolymerization and severing protein⁴² as well as the Arp2/3 complex⁴³ to affect spermatid and phagosome transport across the seminiferous epithelium during the epithelial cycle. This possibility must be carefully evaluated in future studies.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

As briefly discussed herein, plastins are a family of novel actin bundling proteins in which plastin 3 is recently shown to play a crucial role in maintaining the actin microfilament bundles in Sertoli cells, conferring the ES its ability to facilitate the transport of developing spermatids during spermiogenesis, and also other organelles (e.g., phagosomes) during the epithelial cycle of spermatogenesis. Based on the KO studies using genetic models as noted in **Table 1**, it is of interest to assess the phenotypes following a triple KO of all three members of the plastin protein family in the rodent testis since it is likely that other members of the plastin family could supersede the lost function of a plastin family member. Furthermore, it is known that activation of plastins that unleashes its intrinsic bundling activity requires its phosphorylation. For

instance, plastin 2 has two phosphorylation sites near its N-terminus, including Ser-5 (primary phosphorylation site) and Ser-7, and it was shown that phosphorylation of plastin 2 at Ser-5 promoted its targeting to an actin microfilament and to enhance its intrinsic bundling activity,⁴⁴ as well as T cell activation.^{45,46} An impairment of plastin 2 phosphorylation at Ser-5 was recently shown to perturb T cell activation through a disruption of the contact zone between T cells and antigen-presenting cells (APCs) known as the immune synapse.⁴⁷ In studies using HEK294T cells (a human embryonic kidney cell line), PKA, but not PKC, was shown to be the kinase that phosphorylated and activated plastin 2.⁴⁴ Thus, it is of interest and important to determine if PKA (or another protein kinase(s), such as cSrc, cYes, or FAK which are components of the ES⁴⁸⁻⁵⁰) at the ES is responsible for plastin activation in the testis. There are also other outstanding questions remain. Are different protein kinases involved in plastin activation at different stages of the epithelial cycle via their spatiotemporal expression during the epithelial cycle? Are different kinases being used to activate plastin 1, 2 versus 3 in the testis? The answers to these questions may provide important insightful information on the involvement of plastins and also other actin bundling proteins in spermatogenesis, in particular, the signaling pathway(s) that activate plastins along the seminiferous epithelium during the epithelial cycle.

AUTHOR CONTRIBUTIONS

NL and CYC researched on the topic and wrote the first draft; NL and CYC prepared all figures; NL, CKCW and CYC critically evaluated published findings discussed in the paper; CYC prepared the final version; NL, CKCW and CYC read and approved the final version.

COMPETING FINANCIAL INTERESTS

All authors declare no competing interests.

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