Ezrin: a regulator of actin microfilaments in cell junctions of the rat testis

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Ezrin, radixin, moesin and merlin (ERM) proteins are highly homologous actin-binding proteins that share extensive sequence similarity with each other. These proteins tether integral membrane proteins and their cytoplasmic peripheral proteins (e.g., adaptors, nonreceptor protein kinases and phosphatases) to the microfilaments of actin-based cytoskeleton. Thus, these proteins are crucial to confer integrity of the apical membrane domain and its associated junctional complex, namely the tight junction and the adherens junction. Since ectoplasmic specialization (ES) is an F-actin-rich testis-specific anchoring junction—a highly dynamic ultrastructure in the seminiferous epithelium due to continuous transport of germ cells, in particular spermatids, across the epithelium during the epithelial cycle—it is conceivable that ERM proteins are playing an active role in these events. Although these proteins were first reported almost 25 years and have since been extensively studied in multiple epithelia/endothelia, few reports are found in the literature to examine their role in the actin filament bundles at the ES. Studies have shown that ezrin is also a constituent protein of the actin-based tunneling nanotubes (TNT) also known as intercellular bridges, which are transient cytoplasmic tubular ultrastructures that transport signals, molecules and even organelles between adjacent and distant cells in an epithelium to coordinate cell events that occur across an epithelium. Herein, we critically evaluate recent data on ERM in light of recent findings in the field in particular ezrin regarding its role in actin dynamics at the ES in the testis, illustrating additional studies are warranted to examine its physiological significance in spermatogenesis.

Keywords: blood-testis barrier; ectoplasmic specialization; ezrin; spermatogenesis; testis

INTRODUCTION

Ezrin, radixin, moesin (ERM) together with merlin (moesin/ezrin/radixin-like protein, a tumor suppressor, also known as schwannomin or neurofibromin 2) belong to a family of structural proteins called ERM-merlin that cross-link actin filaments of the actin-based cytoskeleton to the plasma membrane, they also create a scaffold for signaling molecules that are involved in the regulation of cell proliferation, migration, and survival.1–4 Studies have shown that ERM proteins are also involved in tumorigenesis due to their involvement in tumor cell migration, such as metastasis. Ezrin is the protein originally identified as band 4.1 detected on Coomassie blue-stained gels when first extracted from erythrocyte plasma membrane.5 Subsequent studies have shown that all ERM proteins share a conserved domain known as band 4.1/ERM (FERM) domain, which is also found in several cytoskeletal-associated proteins, such as focal adhesion kinase (FAK), myosins (e.g., myosin VIIa, X, XV), talins, guanine-nucleotide-exchange factors (GEFs)6,9 (Figure 1). These proteins (e.g., FAK, talin, GEF and myosin) thus interact with ERM-merlin proteins via their FERM domains. While ERM-merlin proteins are highly homologous proteins and ERM proteins have similar binding partners (Figure 1) as well as subcellular localization in the mammalian body, however, ERM display different tissue-specific expression patterns: ezrin is expressed mostly in polarized epithelial and mesothelial cells,10,11 radixin in hepatocytes,12,13 moesin primarily in endothelial and lymphoid cells,10,14 and merlin in nervous tissue.15,16 These proteins are also concentrated abundantly to the undercoat of the plasma membrane of microvilli in the corresponding cells and/or tissues. Thus, ezrin is also known as cytovillin or villin-2. In short, ERM proteins tether integral membrane and cytoplasmic proteins (e.g., adaptors, nonreceptor protein kinases, phosphatases) to actin filaments of the actin-based cytoskeleton, and they also organize apical membrane domain, including tight junction (TJ) and the underlying adherens junction (AJs) of the junctional complex in both epithelia and endothelia.

Interestingly, the knockout (KO) of each of the ERM proteins leads to different phenotypes. In ezrin−/− mice, neonates are normal at birth, but they fail to survive past weaning at ~21-day postpartum (dpp) due to defects in the epithelium of the small intestine, in which epithelial cellular structures that mediate communication between cells are defective, yet cell polarity is unaffected and remains intact,11 illustrating radixin and moesin fail to supersede the lost function of ezrin (Table 1). These observations are important illustrating ezrin is crucial to confer structural integrity of the epithelium in the small intestine to allow proper food absorption. Ezrin may also be crucial to confer epithelial cell communication to coordinate cellular responses of an epithelium as a whole in response to changes in the environment, growth and development, and perhaps pathogenesis.
Radixin<sup>−/−</sup> mice are viable but displaying signs of liver injury by 8-week of age, become hyperbilirubinemia due to defects in the localization of Mrp2 (multidrug resistance-related protein 2, an efflux drug transporter) at the bile canalicular membranes where radixin is concentrated in wild-type normal mice, illustrating radixin is necessary to support the cellular localization of drug transporter Mrp2 for proper secretion of conjugated bilirubin (Table 1). However, moesin-deficient mice are viable without gross abnormalities, both male and female moesin<sup>−/−</sup> mice are fertile, and the cellular localization and expression of ezrin and radixin are not affected in cells and/or tissues<sup>14</sup> (Table 1). But careful analysis of moesin<sup>−/−</sup> mice in a subsequent study have revealed defects in hepatic stellate cells (HSCs) in the liver where moesin is also expressed, in which HSCs fail to respond to liver injury as illustrated by reduced cell migration, and reduced matrix protein (e.g., collagen) deposition during liver injury, developing fibrosis around the injury area, suggesting that moesin may be a target of chronic progressive fibrosis. Merlin is encoded by a gene called neurofibromatosis type 2, a tumor suppressor gene, its inheritable heterozygous mutation in humans leads to the development of multiple nervous system tumors such as Schwann cell tumors.<sup>20</sup> Merlin<sup>−/−</sup> mice died by E6.5–E7.0 due to abnormal development of extraembryonic structures and failure of gastrulation.<sup>21</sup> Furthermore, its deletion in mouse embryo fibroblasts leads to cadherin-mediated AJ destabilization, promoting tumorigenesis and metastasis.<sup>22</sup> These findings thus illustrate the physiological significance of these ERM-merlin proteins, they also illustrate that ERM proteins are not redundant proteins functionally, since a loss of one of these proteins cannot be superseded by other members of the ERM-merlin family.

**Table 1: Functional characterization of ezrin, radixin, moesin and merlin in mammalian tissues and organs**

| Gene name      | Molecular weight (Mr) | Functions                                                                 | Localization                                                                 | General effects in global KO mice                                                                 | Specific effects in conditional KO or RNAi-mediated knockdown | References          |
|----------------|-----------------------|---------------------------------------------------------------------------|----------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|----------------------------------------------------------------|--------------------|
| Ezrin (also known as cytovilin or villin-2) | 85 kDa                | Regulation of actin cytoskeleton, control of cell shape, adhesion and motility and modulation of signaling pathways; epithelial organization; villus morphogenesis; actin polymerization | Cell surface structures, apical microvilli, filopodia, ruffling membranes, retraction fibers, cleavage furrow of dividing cells; adhesion sites where actin microfilaments are associated with the plasma membrane; small intestine; stomach; testes (e.g., spermatids, Sertoli cells) | Neonates are normal at first, fail to survive at~21-day postpartum, abnormal morphogenesis in the gastrointestinal epithelia resulting in postnatal death, impaired cell adhesion and cell motility in small intestine epithelium | Defects in Sertoli cell and spermatid adhesion in the rat testis, knockdown of ezrin perturbs the Sertoli cell TJ-permeability barrier function | 10,11,26,27,59-61 |
| Radixin        | 81 kDa                | Necessary to support cellular localization of Mrp2 for the secretion of bilirubin | Hepatocytes, small intestine, liver, mouse testis                            | Viable, mice are fertile; liver injury by~8 weeks of age due to defects in the localization of Mrp2 in bile canalicular membranes, failing to pump conjugated bilirubin out of the body |                                                                                                           | 12,13,17,26,62,63 |
| Moesin         | 75 kDa                | Lymphocyte homeostasis which include cell shape changes and migration; T-cell activation and polarity | Endothelial and lymphoid cells, HSC, small intestine, lung, liver, uterus    | Viable; no obvious abnormalities, mice are fertile; attenuated responses to liver injury, impaired stellate cell migration and decreased fibrosis |                                                                                                           | 19,26,59,64,65   |
| Merlin (also known as neurofibromin 2, NF2 protein, or schwannomin) with isoform-1 and isoform-2 | 66-70 kDa             | To control the distribution of some membrane receptors, apical integrity and spindle orientation; a tumor suppressor by regulating TJ and AJJs, as well as cortical distribution of ezrin | Epithelia, mesothelial and endothelial cells in humans and mice | Heterozygous mutation leads to NF2 familial cancer syndrome, such as Schwann cell tumor; merlin<sup>−/−</sup> mice died at E6.5–E7.0, failure in gastrulation | Mis-positioned centrosomes, misoriented spindles and aberrant epithelial architecture; deletion of merlin in MEFs destabilizes AJ, promoting tumorigenesis and metastasis | 21,22,66-70     |

**Figure 1:** A schematic drawing to illustrate the functional domains of members of the ezrin, radixin, moesin and merlin (ERM-Merlin) family proteins. All members of the ERM protein family share common structural features of band 4.1 (band 4.1 was designated by Coomassie blue-stained polyacrylamide gel following SDS-PAGE using extracts of erythrocyte plasma membrane<sup>1</sup>), the presence of a band 4.1/ERM domain, an α-helical domain, a proline-rich domain, and an F-actin-binding region in the C-terminal region. ERM-Merlin proteins are activated by phosphorylation at the corresponding Thr or Ser residue.

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| Merlin    | 66-70 kDa             | To control the distribution of some membrane receptors, apical integrity and spindle orientation; a tumor suppressor by regulating TJ and AJJs, as well as cortical distribution of ezrin | Epithelia, mesothelial and endothelial cells in humans and mice | Heterozygous mutation leads to NF2 familial cancer syndrome, such as Schwann cell tumor; merlin<sup>−/−</sup> mice died at E6.5–E7.0, failure in gastrulation | Mis-positioned centrosomes, misoriented spindles and aberrant epithelial architecture; deletion of merlin in MEFs destabilizes AJ, promoting tumorigenesis and metastasis | 21,22,66-70     |
EZRIN IS AN ACTIN-BINDING PROTEIN IN THE MAMMALIAN TESTIS

In the seminiferous epithelium, the ectoplasmic specialization (ES) is an F-actin-rich AJ. The ES is typified by the presence of bundles of actin microfilaments that lie perpendicular to the plasma membrane of Sertoli cells and sandwiched between cisternae of endoplasmic reticulum and: (i) the apposing Sertoli cell-cell plasma membranes, known as basal ES at the blood-testis barrier (BTB) or (ii) the apposing Sertoli cell-spermatid (step 8–19 in the rat testis) plasma membranes, known as the apical ES. These actin microfilament bundles, however, undergo extensive remodeling, switching between their bundled and un bundled/branched configuration to confer plasticity to the ES, so that preleptotene spermatocytes can be transported across the BTB at stage VIII of the epithelial cycle, and developing spermatids can also be transported across the adluminal compartment during the epithelial cycle of spermatogenesis. A study by fluorescence microscopy has detected ERM in the seminiferous epithelium of mouse testes, and ezrin was found to be associated with residual bodies, phagosomes and also apical ES structures. Ezrin was shown to structurally associate with actin but not tubulin in the mouse testis by immunoprecipitation. Ezrin was also found to associate with barbed end nucleation protein actin-related protein 3 (Arp3). Furthermore, ezrin is structurally associated with apical ES protein laminin-γ3, and also basal ES/BTB proteins JAM-A and N-cadherin, supporting the notion that ezrin is an integrated component of the ES based on studies by dual-labeled immunofluorescence analysis. A study using human spermatooza has shown that ezrin is involved in sperm plasma membrane remodeling crucial to sperm capactation. For instance, during sperm capacitation, ezrin was found to be activated at Thr567 (Figure 1) which in turn promoted the formation of cortical cytoskeleton-polymerized actin through Rho activation. Collectively, these findings illustrate the significance of ezrin in the actin microfilament-rich ES.

In the testis, germ cells are known to the connected by intercellular bridges so that their development can be synchronized during spermatogenesis. For instance, preleptotene spermatocytes are connected by intercellular bridges as “clones,” so that their transport across the BTB at stage VIII of the epithelial cycle, and their development to leptotene and zygote spermatocytes can be synchronized during the epithelial cycle until meiosis that takes place at stage XIV in the testis. However, Sertoli cells that provide the structural and nourishment supports to developing germ cells in the seminiferous epithelium are also synchronized during the epithelial cycle. Thus, it remains unknown regarding the mechanism(s) by which Sertoli cells coordinate and communicate with each other between adjacent and distance cells in the epithelium across the seminiferous tubule and also with germ cells during spermatogenesis. Obviously, gap junctions (GJs) play a crucial role in coordinating cellular events in the epithelium during the epithelial cycle by transmitting chemical signals between testicular cells. However, the pore size of GJ communicating channels is limited to small molecules and signaling molecules, at <1.5 kDa in molecular mass, and that GJ is only found between adjacent cells instead of distant cells. Thus, it is likely that larger pore-size channels, such as intercellular bridges, also known as tunneling nanotubes (TNTs), which are actin cytoskeletal-based cytoplasmic extensions that serve as intercellular channels in a number of cell types, are involved in this event. In fact, studies have shown that TNT are tubular connections that allow the transfer of electrical signals, plasma membrane components, pathogens, Ca2+ and even small organelles between adjacent and distant mammalian cells including small regulatory RNAs, crucial in development, tissue regeneration and immunity. In this context, it is of interest to note that earlier findings based on the use ezrin KO and conditional KO mice have illustrated that ezrin is involved in epithelial cell communication to confer the functioning of the intestinal epithelium. Studies in cancer cells and immune cells (e.g., T cells) have illustrated the structural involvement of ezrin in constituting the TNT. A recent report also shows the likely involvement of ezrin in actin organization including its structural association with F-actin in TNT (or intercellular bridges) in Sertoli cells in the rat testis. While intercellular bridges that connect testicular cells such as germ cells have been reported decades ago, and studies have supported the significant role of F-actin in maintaining intercellular bridges, biomolecules that constitute and/or regulate intercellular bridges remain unknown. Recent studies have shown that TEX14 is a critical component of intercellular bridges since its deletion in mice leads to infertility, whereas RBM44 is an intercellular bridge structural protein in the rodent testis. In light of the structural involvement of ezrin in TNT in cancer cells, T-cells, and Sertoli cells, we briefly summarize these recent findings on ezrin, highlighting its likely role in maintaining actin microfilament bundles at the TNT, and illustrating much research is needed in identifying the role of ezrin in TNT/intercellular bridge function.

EZRIN AND SPERMATOCYTOSES – A REGULATOR OF ACTIN MICROFILAMENTS AT THE ECTOPLASMIC SPECIALIZATION

Ezrin was first reported in the mouse testis by fluorescence microscopy, illustrating it is expressed by Sertoli and germ cells, associated with actin microfilaments, involved in spermiogenesis and the maturation of Sertoli cells. Besides expressed predominantly at the Sertoli cell-step 16 spermatid interface in the mouse testis during the epithelial cycle, ezrin is notably expressed in the ultrastructure of residual bodies at stage VIII of the epithelial cycle. In humans, ezrin was found to be associated with spermatozoa, involving in sperm capacitation, possibly due to its role in maintaining the actin-based cytoskeleton and the associated proteins necessary to confer capacitation. A recent study has shown that ezrin is indeed an actin-binding protein in the rat testis, consistent with an earlier report by immunoprecipitation, illustrating ezrin binds to actin microfilaments in the mouse testis. Besides interacting with actin in Sertoli cells (Figure 2), ezrin also binds to Arp3 (which together Arp2 forms the Arp2/3 complex, and when it is activated by neuronal Wiskott–Aldrich Syndrome protein (N-WASP), the Arp2/3-N-WASP complex is known to induce barbed end nucleation, causing branched actin polymerization, effectivity converting actin microfilaments from a “bundled” to an “unbundled/branched” configuration), JAM-A, N-cadherin, c-Src and p-FAK-Tyr. These findings are important since they illustrate the possibility that ezrin, besides an actin-binding protein, it is likely involved in actin microfilament organization during the epithelial cycle to confer plasticity to the ES in the testis, facilitating the transport of preleptotene spermatocytes across the BTB at stage VIII of the epithelial cycle, as well as the transport of elongating spermatids across the adluminal compartment of the seminiferous epithelium during the epithelial cycle so that step 19 spermatids can line-up near to the tubule lumen to prepare for spermiogenesis at stage VIII of the cycle (Figure 3).

This postulate is supported by a study using RNAi to silence ezrin by using ezrin-specific siRNA duplexes versus nontargeting control duplexes. When ezrin was knock-down by ~90%, F-actin organization in Sertoli cells with an established functional TJ-permeability barrier that mimic the Sertoli cell BTB in vivo, was found to be perturbed. For instance, actin microfilaments in Sertoli cells were shown to be grossly disrupted with extensive truncation, which is likely mediated.
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Figure 2: Localization of ezrin in Sertoli cells and the ectoplasmic specialization (ES) in the adult rat testis. (a) Ezrin (red fluorescence) was detected in Sertoli cells and co-localized with F-actin (green fluorescence), at least in part, and appeared as orange-red fluorescence in Sertoli cells when cultured at low cell density (0.5 × 10^5 cells cm⁻²) for 4 days using a specific anti-ezrin antibody as described.27 Scale bar, 80 µm, which applies to other micrographs. (b) Localization of F-actin and ezrin in Sertoli cells when cells were cultured at a cell density of 0.5 × 10^5 cells cm⁻² for 4 days as detailed elsewhere.27 It is noted that ezrin is not completely colocalized with actin microfilaments in Sertoli cells cultured in vitro. Scale bar, 5 µm which applies to other micrographs. (c and d) Ezrin partially co-localized with F-actin at the apical ES at the Sertoli-spermatid interface (d), as well as basal ES/blood-testis barrier proteins N-cadherin and occludin, and F-actin at the Sertoli cell-cell interface (d). Scale bar, 18 µm in (c), 35 µm in top 2 panels in (d), and 18 µm in last panel in (d), which applies to other micrographs in the the same panel.

via a mislocalization and down-regulation of palladin,37 which an actin cross-linking and bundling protein in the testis.31 These changes thus destabilized adhesion proteins at the Sertoli cell-cell interface, such as N-cadherin, which used actin for attachment, as such, the Sertoli cell TJ-permeability barrier function was disrupted.27

The changes that were observed using the Sertoli cell in vitro model as summarized above have been confirmed by in vivo experiments when ezrin was silenced by transfecting testes with ezrin specific siRNA duplexes versus nontargeting control duplexes. For instance, it was shown that the knockdown of ezrin in the testis in vivo indeed perturbed the transport of spermatids and residual body-containing phagosomes in the seminiferous epithelium during the epithelial cycle. For instance, step 19 spermatids were found to be embedded near the basement membrane of the epithelium in both stage VIII and IX tubules.27 Furthermore, phagosomes were also found at the adluminal edge of the tubule lumen in stage IX tubules when they should have been transported to the base of the epithelium for lysosomal degradation.27 These disturbances thus reflect a disruption of the cytoskeleton following the knockdown of ezrin in the testis. Furthermore, the knockdown of ezrin in the testis in vivo also perturbed the integrity of the BTB,27 illustrating ezrin is more than just a structural component of the ES, but a crucial regulator that confers actin dynamics. If ezrin exerts its function simply as an anchoring junction scaffold, its lost could have been superseded by other scaffolding proteins, such as catenins, afadins, and zonula occludens. It is likely that the ezrin is working with other adaptor/signaling proteins at the actin-rich ES to perform essential signaling functions, such as FAK and its activated isoforms.52–54 While the precise molecular mechanism(s) by which ezrin modulates actin dynamics at the ES during spermatogenesis remain to be elucidated, we hypothesize that that ezrin is working in concert with branched actin polymerization-inducing protein Arp2/3 complex, actin cross-linking/bundling protein palladin, and apical ES regulatory protein p-FAK-Tyr²⁹⁵ to maintain the dynamic organization of actin microfilaments at the ES, in particular the apical ES at the Sertoli-spermatid interface, involving in the conversion between the “bundled” and “un-bundled/branched” configuration.35,56 This hypothesis is shown in the schematic drawing in Figure 3. These changes in the organization of actin microfilaments at the apical ES also facilitate endocytic vesicle-mediated protein trafficking events of endocytosis, transcytosis and recycling to facilitate the transport of spermatids, organelles (e.g., phagosomes) and biological substances (e.g. biologically active fragments of laminin chains) during the epithelial cycle (Figure 3). For instance, "old" apical ES proteins derived from the degenerating apical ES at early stage VIII can be used to assemble “new” apical ES when step 8 spermatids arise at this stage of the epithelial cycle. Nonetheless, this model will be updated in the years to come as more data are available in the literature.

It is also noted that ezrin appears to be a component of TNT, namely intercellular bridges, between Sertoli cells, which can be readily detected by immunofluorescence microscopy when Sertoli cells are cultured at low cell density, such as at 5 × 10⁴ cells cm⁻² (Figure 2).27 When ezrin was knockeddown by RNAi to silence its expression by ~90%, the establishment of TNT was shown to be disrupted,27 illustrating ezrin may be crucial for the assembly and/or maintenance of TNT. However, much research is needed to address the role of ezrin in TNT function, in particular in vivo if the loss of ezrin by knockdown would impede signal transfers across TNT.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

While a genetic model of ezrin KO in the mouse is available for a decade,11 its precise physiological role, in particular how ezrin coordinates with other proteins to modulate the TJ and anchoring junction function to regulate tissue barriers, such as the gut barrier, and the F-actin-rich ES in the testis remains unknown. This, by and large, is due to the limitation of using genetic models since the KO of ezrin in rodents makes it difficult to assess its mechanistic functions in particular how the protein exerts its function via its interactions with its partners at the molecular level since the ezrin protein per se is no longer expressed in these mice. Also, ezrin⁻/⁻ died by ~21 dpp, making it not possible to examine its function in adult testes. With recent advances in RNAi, both in vitro and in vivo, we can gain some insightful information on the mechanistic function of ezrin as summarized above. However, much research is needed to better understand the role of ezrin, in particular how it interacts with other actin regulatory proteins to modulate actin microfilaments at the ES during spermatogenesis. For instance, does ezrin serve as a scaffold for p-FAK-Tyr²⁹⁵ so that the ezrin-p-FAK-Tyr²⁹⁵ act as a signaling platform to induce crucial signaling function to regulate apical ES dynamics? Since ezrin was shown to associate with c-Src,25 does ezrin serve as a central coordinator to recruit c-Src to interact with p-FAK-Tyr²⁹⁵ to create a dual-signaling complex to regulate ES dynamics? Furthermore, it was shown that ezrin also associated with Arp3 in the testis,27 does p-FAK-Tyr²⁹⁵ and/or c-Src play a role in modulating the intrinsic activity of either Arp2/3-N-WASP complex or palladin? If ezrin is indeed a crucial regulator to assemble TNT, what is the function of TNT in adult testes? Can TNT in the testis transport chemical signals between distant cells during the epithelial cycle?
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