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Polarity Protein Complex Scribble/Lgl/Dlg and Epithelial Cell Barriers

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Abstract

The Scribble polarity complex or module is one of the three polarity modules that regulate cell polarity in multiple epithelia including blood-tissue barriers. This protein complex is composed of Scribble, Lethal giant larvae (Lgl) and Discs large (Dlg), which are well conserved across species from fruitflies and worms to mammals. Originally identified in *Drosophila* and *C. elegans* where the Scribble complex was found to work with the Par-based and Crumbs-based polarity modules to regulate apicobasal polarity and asymmetry in cells and tissues during embryogenesis, their mammalian homologs have all been identified in recent years. Components of the Scribble complex are known to regulate multiple cellular functions besides cell polarity, which include cell proliferation, assembly and maintenance of adherens junction (AJ) and tight junction (TJ), and they are also tumor suppressors. Herein, we provide an update on the Scribble polarity complex and how this protein complex modulates cell adhesion with some emphasis on its role in Sertoli cell blood-testis barrier (BTB) function. It should be noted that this is a rapidly developing field, in particular the role of this protein module in blood-tissue barriers, and this short chapter attempts to provide the information necessary for investigators studying reproductive biology and blood-tissue barriers to design future studies. We also include results of recent studies from flies and worms since this information will be helpful in planning experiments for future functional studies in the testis to understand how Scribble-based proteins regulate BTB dynamics and spermatogenesis.

Introduction

In invertebrates and vertebrates, epithelial tissues are arranged as sheets of cells that segregate and protect an organism from changes in its environment. In short, epithelial cells are arranged side-by-side with the apical region of their plasma membranes facing the environment, while the basal area is anchored to the basal lamina (i.e., extracellular matrix, also known as basement membrane in the testis^{1,2}), and the lateral area in contact with each other. This feature is achieved via the development of “polarity” during embryogenesis. Thus, epithelial/endothelial cells of an epithelium/endothelium can be divided into three

discrete domains: the apical, the lateral, and the basal domains. Furthermore, at the cell-cell and the cell-extracellular matrix interface, there exist a series of junction complexes. It is noted that an important function of the epithelial barrier is to limit paracellular diffusion from the outside (e.g., environment, blood) to the inside, and this is conferred by tight junctions (TJs) in vertebrates or septate junctions (SJs) in invertebrates. Adhesion between neighboring epithelial cells is conferred by adherens junctions (AJs) lying immediately behind TJs.^{3,4} Therefore, AJs maintain the epithelial sheet whereas TJs act as a “gate” that seals the intercellular space and permits selective exchange of substances (e.g., ions, sugars, amino acids, electrolytes) across an epithelium. The selective permeability of epithelial cells also relies on the polarized distribution of specialized channel proteins on their cell membranes.

Establishment of epithelial cell polarity, including the blood-testis barrier (BTB) in the seminiferous epithelium of mammalian testes, is critical for maintaining cell morphology and function. This requires targeting of membrane and peripheral proteins precisely to either apical or basolateral membrane domains.⁵ The asymmetric distribution of proteins in different parts of membranes is an important feature of epithelial cell polarity. Although the signaling network that controls cell polarity has not been elucidated, a number of membrane proteins that regulate cell polarity have been identified based on genetic and biochemical studies in *Caenorhabditis elegans*, *Drosophila melanogaster*, and also in mammals during these past several decades. These polarity proteins are organized into several functional complexes that distribute at different membrane domains, some of which are mutually exclusive, facilitating the formation of the apicobasal axis and the assembly and maintenance of epithelial cell junctions.⁶ The best studied polarity complex thus far is the partitioning-defective (Par) protein complex, which is located at the apical domain and is composed of Par6, Par3 (also known as *Drosophila* Bazooka) and atypical protein kinase C (aPKC).^{7,8} This tripartite complex also associates with the small GTPase, Cdc42.^{9,10} In mammals, Par6 serves as an adaptor, recruiting other proteins to this complex to facilitate TJ assembly (Fig. 1).^{11,12} The other apical polarity module is composed of the transmembrane protein Crumbs (Crb), the cytoplasmic protein Pals1 [protein-associated with Lin-7, also known as *Drosophila* Stardust, an adaptor and a member of the membrane-associated guanylate kinase (MAGUK) protein family] and Patj (Pals1 associated tight junction protein) (Figs. 1, 2).¹³ Recent studies have shown that Crb complex proteins are tumor suppressors besides serving as polarity proteins.^{6,14} In *Drosophila*, there is also a basolateral polarity protein complex known as the Yrt/ Coracle group, which is composed of FERM proteins Yurt (Yrt), Coracle (Cora); and the membrane proteins Neurexin IV (Nrx-IV) and Na⁺, K⁺-ATPase.¹⁵ The Yrt/Cora complex promotes basolateral membrane stability and it also displays negative regulatory interactions with the apical Crb protein module.¹⁵

In the basolateral domain, there is a protein complex composed of three tumor suppressors: Scribble (Scrib), Lethal giant larvae (Lgl) and Discs large (Dlg), which function cooperatively to regulate cell polarity, junction formation and cell growth in epithelial cells (Figs. 1, 2). This is mediated via mutually exclusive interactions between the Scribble-and the Par-based protein complexes (Fig. 2). Although there is no evidence of physical interaction between the three protein components in the Scribble complex except in

Drosophila neuronal synapses, where Scribble associates with Dlg via a protein named GUK holder (Fig. 1),¹⁶ the three genes show strong genetic interaction (e.g., the knockout of either Scrib, Lgl or Dlg leads to similar phenotypic changes in mutants versus the wild-type) and these proteins appear to utilize a common pathway to regulate cell architecture and cell proliferation.¹⁷ Mutations of these three genes also display similar phenotypes in different epithelia, suggesting that they are components of a fundamental machinery that creates the distinctive architecture of epithelial cells and tissues. In this chapter, we first summarize biochemical features of Scribble complex components. We next focus on their cooperative but distinct roles in regulating epithelial cell junctions and polarity in blood-tissue barriers. We also discuss recent findings on this complex in regulating epithelial barrier functions in different tissues in particularly the BTB in the testis.

Identification and Molecular Structure Of Scribble Complex Components

Members of Scribble complex contain multiple protein–protein interaction domains, in particular PDZ, SH3, and guanylate kinase domains, indicating they can recruit a complex network of proteins to regulate polarity and other cellular functions (Fig. 1). Herein, we focus on the molecular structural features of Scribble, Lgl and Dlg and their functions.

Dlg

The *Drosophila* discs large tumor suppressor gene, *dlg* (lethal discs-large), was first discovered in 1972.¹⁸ Mutations of this gene were found to display a phenotype similar to that of the *lgl* mutation.^{18,19} *dlg* mutants were found to have imaginal discs fused with each other and to the larval ventral ganglion as they overgrew versus the wild type.²⁰ *dlg* was shown to be crucial for normal epithelial structure and growth in the embryo,²¹ indicating the function of this gene is not limited to imaginal discs. Dlg was identified as a member of the membrane-associated guanylate kinase homolog (MAGUK) family, which often locates at cell junctions and contains distinct peptide domains namely PDZ1-3, SH3, HOOK, and GUK (Fig. 1).^{22,23} Studies using different Dlg derivatives have shown that the subcellular targeting of Dlg to the plasma membrane requires PDZ2 and HOOK domains, leading to the precise localization of Dlg at SJs.²¹

To date, five mammalian homologs of *Drosophila* Dlg have been identified (Table 1), with Dlg1 being the best studied Dlg member. The human homolog of *Drosophila* discs large called *hdlg1* was found to contain a C-terminal GUK domain, an SH3 domain, a HOOK domain and three PDZ domains.²⁴ In addition, Dlg1 contains a L-27 domain at its N terminus which can bind to membrane-palmitoylated proteins (MPPs), Lin2/CASK, and Lin7.²⁵⁻²⁸ In vitro study has shown that PDZ1-2 or HOOK domains of hDlg can function independently to localize exogenous hDlg to the basolateral membrane of cells.²⁹

The three homology repeats (PDZ domains) in Dlg were first reported in PSD-95 protein from rat forebrain,³⁰ which was also found in the mammalian TJ protein ZO-1.³¹ Thus, the name PDZ domain (PSD-95, Discs-large, ZO-1) was used to reflect the origin and distribution of this domain.³² In *Drosophila*, Dlg colocalizes with Scribble via a protein named GUK holder, which can interact with both GUK domain of Dlg and the PDZ2 domain of Scribble (Fig. 1).¹⁶

Scribble

Scribble was initially identified in *Drosophila* where it was found to be a crucial regulator of morphogenesis.³³ There is only one *Drosophila* homolog of Scribble in mammals known as Scrib or Vartul (Table 1).³⁴ Scribble is a LAP [LRR (leucine-rich repeats) and PDZ (PSD-95/Discs-large/ZO-1) domain] protein containing 16 LRRs and 4 PDZ domains (Fig. 1). The first LAP protein, Densin 180, which contains a set of LRRs as well as a PDZ domain, was first isolated in rat brain.³⁵ "LAP" is a collective name³³ referring to proteins that contain the LRR and PDZ domains found in fruitflies, worms, rodents and humans.^{33,36,37} The LAP family has three subfamilies according to the number of PDZ domains, namely LAP0, LAP1 and LAP4.³⁸ The LRRs and PDZ domains of LAP proteins are implicated in mediating protein-protein interactions. Due to their unique polarized localization within the cell membrane, LAP proteins play a key role in regulating subcellular protein distribution.³³

LAP family members are strikingly conserved from *Drosophila* to humans, with respect to their primary amino acid sequence, subcellular localization (basolateral in epithelial cells or postsynaptic in neurons) and protein architecture, suggesting common biological functions.³³ A structure-function correlation study on *Caenorhabditis elegans* LET-413, human Erbin and human Scribble (hScrib) revealed that the LRR domain and the LAPSD (LAP specific domain, a 38-amino acid LRR-like domain located between LRR and PDZ domain) are crucial for anchoring members of this protein family to the plasma membrane.³⁹ In short, LRR domains are necessary and sufficient to attach Scribble to the plasma membrane, but it is necessary to have the LAPSD domain to organize a polarized epithelium.⁴⁰

PDZ domains are composed of 80-90 amino acids, folded into two α helices and six β strands that interact with C-terminal sequences of other proteins.⁴¹ Because PDZ domains are frequently found in proteins that regulate cell polarity, it is expected that these motifs are involved in epithelial polarization. However, domain analysis in Scribble has shown that PDZ domains are not indispensable for forming an epithelium, yet they enhance the ability of the LRR to localize proteins to SJs, and to regulate cell proliferation.⁴⁰

Lgl

The *Drosophila* tumor suppressor gene, lethal (2) giant larvae (*l(2)gl*), was first discovered in 1930,^{42,43} and *lgl* was cloned and sequenced in the 1980s.⁴⁴⁻⁴⁶ Mutation of *lgl* in *Drosophila* caused the imaginal discs to overgrow, lose their epithelial structure and the ability to differentiate, forming solid tumors that continued to grow until the death of larvae.¹⁸ *lgl* encodes a 130-kDa protein that contains stretches of sequences that are similar to cell adhesion proteins.⁴⁵ Homologs of Lgl have been identified in a variety of organisms, all of which share conserved primary sequences.⁴⁷ It has been demonstrated that mammalian Lgl proteins (Lgl 1-4, Table 1) contain at least 4-5 putative WD-40 repeat motifs⁴⁸ (Fig. 1). WD-repeats, each of which comprises a four-stranded anti-parallel β sheet,^{49,50} are known to contain minimally conserved domains of ~40-60 amino acids that begin with a glycine-histidine (GH) dipeptide 11 to 24 residues from the N terminus and end with a tryptophan-aspartic acid (WD) dipeptide at the C terminus.⁵¹ WD-repeat proteins often function as coordinators of macromolecular protein complexes, such as for protein-protein interaction,

receptor-ligand interaction in signal transduction,⁵²⁻⁵⁴ pre-mRNA processing,^{55,56} cell cycle regulation,⁵⁷ and cytoskeleton assembly.⁴⁷ Recent studies have illustrated the cellular function of WD-40 repeat motif in Lgl family members. For instance, WD-40 repeats are implicated in promoting the binding of Lgl with the LRR domain of Scribble.⁵⁸

Relationship Between Cell Junctions and Cell Polarity In Epithelial Cells

Epithelial cell polarity refers to the asymmetric distribution of cellular organelles of different structures and/or functions between the apical and the basolateral domains. This apical-basolateral polarization is essential for epithelial cell function, including cell division (mitosis and meiosis), cell migration, and molecular transport,⁵⁹⁻⁶¹ and it is intimately related to TJ and AJ dynamics at the cell-cell interface.

TJs that create the limit of the apical and the basolateral domains are composed of three types of proteins: occludins, claudins and junctional adhesion molecules (JAMs). These TJ proteins all bind to the N-terminal PDZ domains of zonula occludens (ZO-1, -2 and -3), members of the MAGUK (membrane-associated guanylate kinase-like homology) family, via their cytoplasmic tails. Since TJs prevent diffusion of membrane proteins between the apical and the basolateral regions in a cell epithelium, it serves as a “fence” or a “barrier” that leads to epithelial cell polarity.

AJs are located immediately underneath TJ in virtually all epithelial barriers.^{4,62,63} AJs are formed by homophilic interactions between extracellular domain of cadherins and nectins. The cytoplasmic tails of cadherins and nectins associate with a series of intracellular peripheral proteins (e.g., catenins and afadins, which are adaptors), which take part in different biological events, including cytoskeleton organization⁶⁴⁻⁶⁶ and regulation of gene expression.^{67,68} The interactions between functional polarity complexes, the apical Par- and the Crumb-based modules, and the basolateral Scribble module, generating zones of mutual exclusion around AJs that define the apicobasal axis of epithelial polarity.⁶

The actin-based cytoskeletal remodeling at the AJ is regulated by Rho, Rac and Cdc42 GTPases.⁶⁹ However, interactions between AJs and Rho GTPases are bidirectional since AJs also modulate the intrinsic activity of Rho GTPases to adjust cell structure and polarity. Cdc42 is a regulator of actin cytoskeleton and is associated with Par3-Par6-aPKC polarity complex.¹¹ Cdc42, together with Par proteins, were found to regulate AJ stability via their effects on protein trafficking, such as protein endocytosis, in *Drosophila melanogaster* neuroectodermal epithelium, illustrating functional interactions between cell polarity proteins and endocytosis that are critical to stabilize basolateral AJs.⁷⁰ Recent studies in the testis have shown that Cdc42 is a component of the Par6-based polarity complex.^{71,72} Cdc42 is crucial to BTB remodeling during spermatogenesis by regulating endocytic vesicle-mediated protein trafficking,⁷¹ since overexpression of a dominant negative mutant of Cdc42 in Sertoli cells that blocks the Cdc42 function can lead to a loss of the ability of TGF- β 3 to accelerate protein endocytosis at the Sertoli cell BTB,⁷¹ thereby promoting the Sertoli cell TJ-barrier integrity.^{73,74} Earlier studies have shown that cytokines (e.g., TGF- β 2, TGF- β 3) are potent regulators of BTB dynamics by perturbing TJ-barrier function, which is mediated via their effects in accelerating the kinetics of clathrin-mediated endocytosis of

integral membrane proteins (e.g., N-cadherin, occludin) at the Sertoli cell BTB,^{75,76} as well as their subsequent endosome-/ubiquitin-mediated degradation.^{76,77}

Scribble Complex in Regulating Cell Junction Dynamics and Cell Polarity

Mutations in *scribble* were found to cause aberrant cell shapes and loss of epithelial organization in *Drosophila* embryos.³³ Interestingly, mutation in either Lgl or Dlg led to similar phenotypic changes in epithelia *versus* Scribble mutant.¹⁷ Furthermore, Scribble and Dlg colocalized and overlapped with Lgl in embryonic epidermal epithelium, and *scribble*, *dlg* and *lgl* displayed strong genetic interactions.¹⁷ These findings thus indicate that these three tumor suppressors share a common role in regulating epithelial polarity and cell growth.

Dlg in Regulation of Cell junctions and Cell Polarity

The product of the *Drosophila* tumor suppressor gene *dlg* is a member of the MAGUK family, which is located at the cytoplasmic face of SJs and is necessary for the formation of cell junctions and the maintenance of apico-basal polarity.^{22,78,79} In *Drosophila*, the complete loss-of-function of *dlg* allele (*dlg^{m52}*) was shown to induce: (i) rearrangement of the cytoskeletal components (e.g., actin and tubulin) and proteins having a polarized distribution (e.g., Ex, Cor), (ii) mis-localization of transmembrane proteins (e.g., fasciclin III, neuroglian), (iii) loss of SJs and growth control, and (iv) a loss of ability to differentiate.⁷⁹

In mammals, hDlg1 (human homolog of Disc large) colocalizes with E-cadherin at the cell-cell contact site in intestinal and renal epithelial cells.⁸⁰ A knockdown of hDlg in intestinal epithelial cells by siRNA severely alters AJ integrity and also prevents the recruitment of p85/PI3K to E-cadherin-based cell-cell contact,⁸⁰ which is required for the association of AJ components with the cytoskeleton and also for AJ assembly.⁸¹ This is consistent with findings in *C. elegans* which illustrated a disrupted AJ associated with a mis-localization of AJM-1 (apical junction molecule, an AJ marker) in epidermis and intestinal epithelial cells after Dlg1 knockdown.⁸² In short, hDlg1 is a crucial regulator of AJ and TJ assembly in mammalian epithelia, even though the underlying regulatory mechanism(s) remains known.

Scribble in Regulation of Cell Junctions and Cell Polarity

Mutations in *Drosophila scribble* led to embryos displaying corrugated cuticular surface which was riddled with holes, while the wild type embryonic cuticle formed a smooth, continuous sheet, and hence the name *scribble* was used.³³ Scribble is located basal to Armadillo (Arm, the homolog of vertebrate β -catenin) in the epithelial SJs, and codistributes with Coracle (a SJ marker belongs to protein 4.1 family).³³ Thus, Scribble localizes to epithelial SJs in *Drosophila*, and restricted to the boundary of the apical and basolateral cell surfaces. In vertebrates, however, Scribble is distributed along the entire basal and lateral domains of epithelial cells (Figs. 1 and 2). In embryonic epithelial cells of *C. elegans*, the homolog of Scribble, Let-413, also localizes in the basolateral region and it is involved in locating AJ components properly at a discrete subapical position.^{37,83} For instance, a loss of *scribble* (or *let-413* in *C. elegans*) rendered a disorganization of epithelia, leading to a mis-

distribution of apical proteins (e.g., Arm, Sas, Dlt, and Crb) and AJ proteins (e.g., E-cadherin in *Drosophila*, and HMP-1, JAM-1 in *C. elegans* embryos) with these proteins moved to the basolateral cell surface, but the localization of basolateral proteins was found to be unaffected.^{33,83} This preferential loss of apical protein restriction that leads to polarity defects in *scrib* mutant epithelial cells is the result of mis-distribution of apical proteins.

Although Scribble, together with Dlg,¹⁷ are localized to SJs in *Drosophila*, no protein-protein interaction between either one of these proteins with SJ components was found. During development, Scribble and Dlg are enriched in the membrane before the appearance of SJs,⁸⁴ so it was assumed that Scribble might predetermine the site of future SJs by recruiting other SJ components to the site for its assembly.⁴⁰

The biological roles of mammalian Scribble (Scrb1, hScrib) are largely unknown. However, hScrib was localized to cell junctions of MCF10-2A, Caco-2 and MDCK cells, colocalizing with β -catenin.⁸⁵ hScrib-depleted MCF10A or MDCK cells did not lead to cell polarity defects as seen in *Drosophila*.^{86,87} However, hScrib knockdown in MCF10A cells exhibited defects in wound closure and chemotactic movement in vitro. For instance, hScrib silenced cells at the wound edge failed to polarize and to recruit Cdc42 and Rac1 to the leading edge, which is required for the formation of polarized lamellipodia and to facilitate subsequent migration.⁸⁶ Also, hScrib mutant mice were found to have defect in epidermal wound healing.⁸⁶ A recent study in mammary epithelial cells demonstrated that depletion of hScrib led to a disrupted 3D acinar morphogenesis by inhibiting the establishment of apicobasal polarity; and, most interestingly, cell apoptosis triggered by activation of *c-myc* oncogene was blocked in these cells.⁸⁸ These results thus illustrate the crucial role of hScrib in regulating cell polarity and perhaps cell apoptosis.

In contrast to flies and worms, mammalian Scribble was found to interact with cell junction proteins. For example, co-expression of hScrib and ZO-2, a TJ adaptor protein and a tumor suppressor,^{89,90} in COS-7 cells has demonstrated a direct interaction between these two proteins, which is mediated by the PDZ3 and PDZ4 domains of hScrib (Fig. 1) and the carboxyl-terminus of ZO-2.⁹¹ In MDCK cells, a knockdown of Scribble was found to disrupt E-cadherin-mediated cell adhesion, with these cells acquiring a mesenchymal appearance, migrating more rapidly but with a loss of directionality.⁸⁷ Interestingly, this adhesion disruption could be partially rescued by overexpressing an E-cadherin- α -catenin fusion protein but not E-cadherin alone. This result thus suggests that Scribble, as a tumor suppressor, it stabilizes the coupling between E-cadherins and catenins, regulating epithelial cell adhesion and migration.⁸⁷

In addition to epithelial cells, mammalian Scribble is likely involved in planar cell polarity (PCP) in mammals (note: PCP originally refers to the directional polarity of hairs on the wings of fruitflies,⁹² analogous to the directional alignment of elongating/elongated spermatids with their heads pointing toward the basement membrane in the seminiferous epithelium during spermatogenesis). The study of planar polarization in mammals has demonstrated the role of Scribble in PCP pathway.⁹³ Mutation of *Scribble* gene in mouse led to defects in the polarization of stereociliary bundles in cochlea, and similar but more severe defects were observed in animals with mutation in *Vangle2*, a mammalian homolog of the

Drosophila PCP gene *Strabismus/Van Gogh*.⁹³ Moreover, polarization defects in animals heterozygous for *Vangl2* and *Scrb1* were comparable to *Vangl2* homozygotes, demonstrating genetic interactions between these two genes in regulating PCP in mammals.⁹³ A subsequent study in mammalian cells has shown that *Scrb1* and *Vangl2* interacted with each other through the PDZ domains of Scribble.⁵⁸ These results illustrate that Scribble is involved in regulating PCP in *Drosophila* and mammals. However, the involvement of Scribble in spermatid alignment in the seminiferous epithelium during spermiogenesis with their heads pointing to the basement membrane but their tails to the seminiferous tubule lumen, which appears to be a form of planar cell polarity, remains to be established. It is likely that the spermatid PCP in the seminiferous epithelium requires the concomitant contribution of PAR-, Crumbs-, and the Scribble-based modules. This possibility must be carefully evaluated in future studies.

Mutual Inhibition of Lgl and Apical aPKC-Par6 in Regulating Epithelial Cell Polarity

Similar to *dlg*, *lgl* was initially identified as a tumor suppressor, because mutation in this gene resulted in tissue-specific tumors in larvae that led to their eventual death.^{94,95} Studies in *Drosophila* embryos have shown that Lgl is localized at cell membranes or intercellular matrix of embryonic cells and it is likely involved in cell-cell interactions.^{45,96} In plasma membrane, Lgl was found to form large aggregates which were resistant to solubilization by nonionic detergents, indicating its involvement in cytoskeletal matrix.⁹⁷ Lgl also forms homo-oligomers and directly interacts with nonmuscle myosin II.⁹⁸ Furthermore, Lgl was found to tightly associate with aPKC in the Par protein complex.⁹⁹ The phosphorylation of Lgl by aPKC leads to a release of Lgl from the plasma membrane, dissociating from nonmuscle myosin II without affecting Lgl homo-oligomerization.¹⁰⁰ Additionally, genetic and phenotypic analyses in mutant *Drosophila* embryos suggested that the *lgl* product was required in different types of epithelial cells to control cell shape during development in vivo.¹⁰¹ Collectively, these studies illustrate the involvement of Lgl in the formation of cytoskeletal network, and its interaction with cell membrane is mediated via its phosphorylation by aPKC.

The mutual inhibition of Lgl and aPKC has been reported in *Drosophila*. When Lgl is phosphorylated on three conserved Ser residues by aPKC, Lgl dissociates from the cytoskeleton and becomes inactivated, which, in turn, disrupts proper protein localization to the apical cell cortex.⁹⁹ Also, phosphorylation of Lgl causes an auto-inhibitory interaction of its N terminus with its C-terminus, which prevents the binding of its C-terminus to the cytoskeleton.¹⁰² On the other hand, aPKC mutants were found to display a phenotype opposite to that of the *lgl* mutants, i.e., a reduced aPKC level suppressed both cell polarity and cell proliferation in *lgl* mutants.¹⁰³ In *Drosophila*, the maintenance of Par6 localization to the apical side of epithelial cells requires Lgl, which is active on the basolateral side and excludes Par6 from the cell cortex. While on the apical side, Lgl is inactivated and excluded by aPKC phosphorylation.¹⁰⁴ These results suggest that mutual inhibition and exclusion of aPKC and Lgl on opposite sides of an epithelial cell participate in the formation of complementary corticle domains and correct epithelial structure.

In vertebrates, the Lgl homologs, Lgl1 and Lgl2, display similar antagonistic interactions with aPKC-Par3-Par6 complex in regulating epithelial cell polarity and proliferation. In contact-naïve MDCK cells caused by the absence of Ca^{2+} in spent medium, cell adhesion and TJ formation were blocked with Lgl accumulated in punctate cytoplasmic structures. After the addition of Ca^{2+} to the medium, Lgl was found to associate with the lateral surface following E-cadherin appearance and TJ formation.¹⁰⁵ This indicates that the engagement of Lgl to the lateral membrane correlates with the development of a polarized structure in mammalian epithelial cells. This lateral domain restriction of mammalian Lgl (mLgl) is dependent on its phosphorylation within a highly conserved region, because a phosphorylation-resistant recombinant is observed to distribute in a nonpolarized manner.¹⁰⁵ In MDCK cells, mLgl competes with Par3 for its interaction with aPKC-Par6 to form a protein complex.¹⁰⁶ During epithelial polarization, mLgl temporally colocalizes with aPKC-Par6 at the cell-cell interface and inhibits the formation of TJs, while another complex, Par3-aPKC-Par6, promotes TJ formation. Importantly, the phosphorylation of mLgl by aPKC is involved in its dissociation with aPKC-Par6 during polarization of epithelial cells.¹⁰⁶ These findings indicate that aPKC/Par6 selectively associates with either Par3 or Lgl, depending on the intrinsic activity of aPKC, to exert its regulatory function on epithelial cell polarity.

Using siRNA knockdown, it was shown that mLgl regulated the assembly of apical membrane domains and cell polarization in MDCK cells.¹⁰⁷ Utilizing the Ca^{2+} -switch model, MDCK cells cultured in Ca^{2+} -depleted medium were found to have reduced levels of peripheral apical proteins and F-actin due to their redistribution from the cell surface to cell cytosol. However, in the mLgl-knockdown cells, the redistribution of these proteins was significantly suppressed and most of the mLgl-knockdown cells retained the apical proteins and F-actin at the cell periphery. Importantly, overexpression of dominant-negative aPKC λ was found to antagonize the effect of the mLgl knockdown on the redistribution of apical proteins.¹⁰⁷ This indicates that in mammalian epithelial cells, aPKC activity also contributes to suppressing the disassembly of apical proteins during cell depolarization, and mLgl counterbalances this aPKC activity to facilitate apical protein disassembly. This study also revealed that the suppression of apical Par3-aPKC-Par6 complex activity by mLgl is mediated through the suppression of an interaction of the aPKC-Par6 complex with Par3 or Cdc42.¹⁰⁷ p32, a mLgl binding protein, was found to form a complex with mLgl and aPKC in regulating cell polarity.¹⁰⁸ Overexpression of p32 could rescue the reduced apical actin filaments caused by aPKC inhibitor whereas its knockdown induced defects in cell polarity.¹⁰⁸ This study thus suggests that p32 regulates cell polarization by binding to mLgl and aPKC and enhancing phosphorylation of mLgl via aPKC. These results thus illustrate that the mutual suppression between mLgl and aPKC-Par6 also plays an important role in the establishment of mammalian epithelial cell polarity.

Interactions between Scribble Complex and Other Apical Polarity Complexes in Epithelia

As briefly described above, several protein complexes that are involved in epithelial apicobasal polarity have been identified. Using genetic and biochemical approaches, details of the protein-protein interactions that connect these complexes are increasingly clear (Fig. 2). For example, the spatial relationship between Crb complex and Par complex is supported by findings in *Drosophila* and mammalian cells.¹⁰⁹⁻¹¹³ Co-immunoprecipitation in MDCK

cells has illustrated direct interaction between Crb3, one of the Crumbs homolog in mammals, and Par6, which is required for the establishment of mature junction structure in epithelial cells.¹⁰⁹ In *Drosophila*, the cytoplasmic tail of Crb that contains two conserved Thr was shown to bind directly to aPKC. Moreover, Dpatj of the Crb complex can regulate the phosphorylation of Crb by aPKC, which induces Dpatj and Scribble to localize to the proper cell membrane domain.¹¹⁰

In addition to the antagonistic interaction between Par3-aPKC-Par6 complex and Lgl, another complex that belongs to apical domain of epithelial cells, the Crb complex, has also been found by genetic screening to have functional interactions with components of Scribble complex in *Drosophila*.^{114,115} Crb is an integral apical protein that plays a role in specifying the apical domain of epithelial cells by forming a complex with Stardust (Sdt) and Dpatj in *Drosophila*.^{116,117} Overexpression of Crb results in the expansion of the apical domain and a decrease in the basolateral domain, which is consistent with the phenotype caused by impaired activity of basolateral components of Scribble complex.^{118,119} Interestingly, genetic study revealed that the epithelial defects caused by *crb* mutation were markedly suppressed by *scribble* mutation.^{114,115} Thus, it is likely that the Scribble complex antagonizes Crb in setting the limits of the apicobasolateral domains and in determining the relative distribution of cell junction proteins in these domains.

While no intrinsic catalytic activity is detected in components of the Scribble complex, several protein-protein interaction domains, such as PDZ, SH3 and GUK domains, are found in their primary sequences, illustrating the likely interactions between Scribble/Lgl/Dlg complex and other proteins, which can affect epithelial polarity and cell proliferation. Using biochemical approaches in human epithelial cells, MPP7 (membrane-palmitoylated protein 7), another member of MAGUK family was identified to interact with hDlg1 via its N-terminal L27 domain, and also with Pals1 (MPP5) and Patj, which are components of the Crb complex, via its SH3-HOOK domain.^{25,120} In Caco2 cells, both hDlg1 and MPP7 displayed partial colocalization with occludin and ZO-1, and shRNA-mediated knockdown of either one of these proteins compromised TJ assembly, indicating the interactions between these polarity complexes affect cell junction function.²⁵ In migrating astrocytes, there is evidence that membrane-associated Dlg1 interacts with microtubule-bound APC (adenomatous polyposis coli, another tumor suppressor) through its PDZ domains to polarize the microtubule cytoskeleton during cell migration, which is required for polarization of astrocytes.¹²¹ Furthermore, PKC ζ -Par6 complex activated by Cdc42 was shown to control microtubule recruitment of APC and cortical recruitment of Dlg1.¹²¹ This suggests a hierarchical biochemical connection exists between the two distinct polarity complexes.

These observations suggest that components of Scribble complex co-operate and compete with the apical polarity proteins to define the apico- and baso-lateral domains by recruiting respective junction proteins to the sites during cell polarity establishment.

Scribble Complex Participates in Cell Signaling Cascades

As summarized above, the Scribble complex and its homologs regulate both cell polarity and cell proliferation. Yet little is known about the underlying mechanism(s) particularly in mammals. However, recent findings have shed lights on the complex signaling network through which Scribble/Dlg/Lgl affects cell polarity and proliferation (Fig. 2).

SAPK3/p38 γ is a one of the p38 MAPKs (p38 mitogen-activated protein kinases) which is activated in mammalian cells in response to hyperosmotic stress.^{122,123} It has a C-terminal sequence that can dock with the PDZ domains of different proteins, one of which is hDlg. Studies by immunofluorescence and co-immunoprecipitation in Hela, PC12 and SH5-SY5Y cells have detected interaction between SAPK3/p38 γ and hDlg, revealing specific phosphorylation of hDlg by SAPK3/p38 γ in vivo in response to cellular stress.¹²⁴ In addition, this phosphorylation of hDlg was found to induce its dissociation from guanylate kinase-associated protein (GKAP), which is necessary for its targeting to cytoskeleton.^{124,125} c-Jun N-terminal kinase (JNK) was also found to induce phosphorylation of hDlg during osmotic stress that contributed to its accumulation within the cell membrane at cell-cell contact site, which was coupled with an increase in E6-induced degradation of hDlg.¹²⁶ These data thus suggest that the cellular localization, function and proteasome-mediated degradation of hDlg are phosphorylation-dependent events.

Furthermore, the involvement of mammalian Scribble in oncogenic transformation was shown to be mediated via a suppression of MAPK signaling.¹²⁷ For instance, simultaneous knockdown of Scribble by shRNA and an activation of Ras-signaling in nontransformed human MCF10A mammary epithelial cells were shown to promote cell invasion.¹²⁷ Moreover, a two-fold increase in the phosphorylation of ERK (extracellular-regulated kinase, an MAPK effector) and an acute hyperphosphorylation of ERK in response to EGF, were also detected in Scribble-depleted cells.¹²⁷ In Scribble-depleted cells, an activation of Raf-MEK-ERK signaling was found to enhance the defects of Scribble loss. Furthermore, Scribble-overexpressed cells were found to suppress the development of invasive protrusions of Ras-activated cells, and Scribble overexpression also facilitated the restoration of cell polarity in Ras-activated cells.¹²⁷ These data thus illustrate that Scribble acts as a tumor suppressor by limiting cell invasion, which is mediated downstream of Ras but upstream of MAPK in the signaling pathway.

A recent study has revealed the likely mechanism by which Scribble suppresses the Ras-Raf-MEK-ERK pathway.¹²⁸ Two ERK binding sites (kinase interaction motif, KIM) were identified on hScrib, and hScrib was found to regulate the ERK signaling pathway in human keratinocytes by inhibiting the phosphorylation and nuclear translocation of ERK.¹²⁸ In vitro study also revealed the two phospho-acceptor sites of hScrib were putative substrates for PKA and ERK1.¹²⁸ These results thus support the notion that a loss of Scribble can affect mitotic proliferation in tumors by promoting MAPK phosphorylation and nuclear translocation of ERK.

In mammalian tissue barriers, there is also evidence that Scribble complex components participate in endocytic vesicle-mediated trafficking and membrane fusion events. For example, membrane-associated mLgl from MDCK cells was found to directly interact with Syntaxin 4 (STX4), a component of the exocytic pathway at the basolateral membrane.¹⁰⁵ Recently, a study in HaCaT cells has shown that three components of the Scribble complex, hScrib, mLgl and hDlg all display significant degree of colocalization with STX4 at cell membrane.¹²⁹

The Scribble Module and Mammalian Epithelial Barrier Function

Epithelial monolayers that display apico-basal cell polarity serve as tissue barriers in multiple organisms. Cell polarity and impermeability are two important attributes of these tissue barriers. The polarity protein complexes described above cooperate with each other to confer differential distribution of functional proteins on plasma membrane. On the other hand, junction complexes formed at correct cortical positions help to maintain distinctive cell membrane domains and resist material exchanges between two sides of the epithelial monolayer. Therefore, the development of cell polarity plays an important role in epithelial barrier establishment and function. In this section, we focus on the role of Scribble polarity complex on the formation and function of several mammalian epithelia and the likely underlying mechanism(s).

Scribble Regulates Barrier function of Intestinal Epithelium

In the small intestine, the gut barrier conferred exclusively by TJs of the intestinal epithelium protects the host animal from invasion of pathogens during food digestion and absorption. Although the causal relationship between the establishment of TJs and cell polarity remains unclear, the role of TJs in sealing the paracellular space that creates the gut barrier has been established.^{130,131}

The role of Scribble in TJ assembly was first demonstrated in T84 and SK-CO15 intestinal epithelial monolayers in which Scribble was found to colocalize with TJ proteins occludin and ZO-1.¹³² This is in contrast to other mammalian epithelia in which Scribble was restricted to the basolateral plasma membrane.^{91,133} Moreover, similar to the findings in Scribble-depleted MDCK cells,⁸⁷ a knockdown of Scribble by RNAi was found to significantly delay gut barrier assembly, and to perturb the TJ-barrier function of Scribble-deficient cells.¹³² Interestingly, Scribble knockdown in SK-CO15 cells did not affect the TJ ultrastructure, nor impeded the development of apicobasal cell polarity.¹³² These data illustrate Scribble is crucial to the gut barrier function in vitro.

In the intestinal epithelium, Scribble was found to interact directly with ZO-1¹³² and ZO-2,⁹¹ which are cytosolic scaffolds that stabilize integral membrane TJ proteins (e.g., occludins, claudins, JAMs). A study to monitor the subcellular distribution of Scribble in mouse small intestine epithelium showed that Scribble was localized to the basolateral membrane. In addition, Scribble was also detected at the site of apical junction complex.¹³⁴ Moreover, a distinctive colocalization of Scribble and E-cadherin was detected at the apical junction site, illustrating an apical and basolateral localization of Scribble in mouse intestinal epithelium.¹³⁴

Although there is little evidence of physical interaction between components of Scribble complex in mammalian epithelia, a recent report in the gut barrier has revealed selective binding between Scribble and Lgl-1, but not Dlg-1, during TJ reassembly.¹³² More important, a knockdown of Dlg-1, but not Lgl-1, was found to attenuate the gut barrier function.¹³² Furthermore, interferon (IFN)- γ , a pro-inflammatory cytokine, was found to induce down-regulation of junctional Scribble during TJ disassembly caused by the IFN- γ -induced intestinal inflammation in vivo.¹³² These findings seemingly suggest the absence of a trimeric Scribble/Lgl/Dlg complex in mammalian intestinal epithelium, and Scribble is likely to play a unique role in gut barrier function.

Scribble Complex Controls Renal Epithelial Polarity

In the kidney, tubular epithelium is composed of polarized epithelial cells with the apical membrane domain facing the tubular lumen, and cell junctions are either restricted to the cell-cell interface or to the cell-basal lamina interface at the basolateral domain. In the kidney, cell polarity and cell junctions in the epithelium of convoluted tubules are necessary to fluid/electrolyte re-absorption and secretion. Studies by confocal microscopy showed that hScrib was located at the cell-cell interface of MDCK (Madin Darby canine kidney) cells, colocalizing with β -catenin. Calcium switch assay also demonstrated hScrib was retained to cell junction upon engagement of E-cadherin by its LRR region in MDCK cells.⁸⁵ Binding of hScrib to E-cadherin at the lateral side is important for normal adhesion between polarized renal epithelial cells, since knockdown of hScrib was found to increase cell motility and reduce adhesion.⁸⁷ mLgl was localized to basolateral membranes and it interacted with basolateral exocytic machinery in MDCK cells.¹⁰⁵ Further studies in renal epithelium also revealed the interaction between mLgl and aPKC-Par6 complex.¹⁰⁶ In hDlg1-deficient mice, abnormalities were found in the renal and urogenital organs. For instance, the kidneys and ureters of hDlg1-deficient mice became hypoplastic due to reduced cell proliferation in the uretic or renal epithelium.¹³⁵ These findings thus illustrate the involvement of Scribble complex in the development, cell proliferation, and maintenance of renal epithelial polarity and integrity.

Scribble Complex and the BTB

Based on studies in the field as briefly reviewed and summarized above, it is increasingly clear that the Scribble/Dlg/Lgl polarity protein module, whose components are tumor suppressor genes, is crucial to cell junction dynamics in mammalian epithelial cells. Furthermore, each of the components in this complex can recruit numerous partner proteins via their protein interacting domains (e.g., PDZ domain). As such, a complex network of proteins can be recruited to specific cellular sites/domains and that this complex can regulate a wide range of cellular functions besides cell polarity. Interestingly, even though components of the Scribble protein complex, namely Scribble, Dlg, and Lgl, were discovered ~3 to 4 decades ago in *Drosophila*, and their mammalian homologs were since identified and shown to possess similar physiological functions, there is no report in the literature that examined their function in the testis, such as the BTB during spermatogenesis. As shown in Figure 3, Scribble, Dlg1 and Lgl2 were shown to be expressed in the testis, with these proteins expressed by both Sertoli and germ cells. Using an antibody specific to Scribble, its localization in the seminiferous epithelium of the rat testis has also been

examined by immunohistochemistry (Fig. 4), illustrating Scribble is localized at the basal compartment of the seminiferous epithelium near the basement membrane, consistent with its localization at the BTB (Fig. 4). More important, the localization and expression of Scribble at the BTB appears to be stage-specific since Scribble is abundantly found at the BTB at Stages VII to VIII of the seminiferous epithelial cycle at the time of BTB restructuring to accommodate the transit of preleptotene spermatocytes at the site (Fig. 4). The localization of Scribble at the BTB has also been confirmed by dual-labeled immunofluorescence analysis, in which Scribble was found to colocalize with putative integral membrane protein occludin and TJ adaptor protein ZO-1 at the BTB (Fig. 5). These findings thus demonstrate unequivocally that Scribble is an integrated component of the BTB. These results also illustrate that functional studies can now be performed to probe the physiological role of the Scribble/Dlg/Lgl at the BTB. Furthermore, it will be important in future studies to examine how these proteins interact with the Par-based polarity proteins, such as Par 6 and 14-3-3, which were shown to regulate spermatid orientation and cell adhesion at the BTB, via their effects on the endocytic vesicle-mediated protein trafficking.^{71,136,137}

Conclusion and Future Perspectives

The Scribble/Dlg/Lgl complex has been shown to regulate cell polarity, TJ and AJ dynamics, and cell proliferation in species ranging from fruitflies, worms and mammals (e.g., the gut barrier, the tubular barrier in the kidney) as summarized above. It is anticipated that this polarity complex is a multifaceted functional complex at other blood-tissue barriers, such as the BTB, where components of this protein module were found. Also, it will be important in future studies to assess its functional relationship with the PAR- and the Crumb-based complexes since PAR-6,¹³⁶ 14-3-3,¹³⁷ and Cdc42⁷¹ were shown to be integrated components of the Sertoli cell that regulated BTB dynamics, and how these three polarity modules fine tune the timely restructuring of the BTB during the seminiferous epithelial cycle of spermatogenesis. For instance, it remains to be examined if the Scribble complex regulates endocytic vesicle-mediated protein trafficking at the BTB; and if it does, how this complex works with the PAR-based protein complex to modulate protein endocytosis, transcytosis, recycling, and endosome-/ubiquitin-mediated degradation.

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Figure 1.

Schematic drawing that illustrates the different functional domains of Scribble complex components in the baso-lateral region versus other apical polarity proteins in the apical region of mammalian epithelial cells. This drawing is also applicable to the testis except that the BTB in the testis lies adjacent to the basement membrane (a modified form of extracellular matrix also known as basal lamina in other epithelia) in the seminiferous epithelium where components of the Scribble protein complex are localized (see Figs. 4 and 5), whereas apical polarity proteins (e.g., Par6) are found both at the Sertoli cell-spermatid interface in the apical region of the seminiferous epithelium and at the BTB.⁷²

Abbreviations used: TJ, tight junction; AJ, adherens junction; PB1, Phox and Bem1p domain; semi-CRIB, semi-Cdc42/Rac interactive binding motif; PDZ, PSD-95/Discs-large/ZO-1 conserved domain; C1, protein kinase C conserved region 1; EGF motif, epidermal growth factor motif; L27, Lin2 and Lin7 binding domain; SH3, Src homology 3 domain; GUK, guanylate kinase homologs conserved domain; LRR, leucine-rich repeats; LAPSD, LAP specific domain; WD, WD-40 repeats.

Figure 2.

Interactions of Scribble complex components with other polarity or signaling proteins in polarized epithelial cells. The information depicted here is applicable to the seminiferous epithelium of mammalian testes where many components of these proteins have recently been identified. Abbreviations used: Patj, Pals1 associated tight junction protein; Pals1, protein-associated with Lin-7; Crb, Crumbs; Par, partitioning-defective proteins; aPKC, a typical protein kinase C; Scrib, Scribble; Dlg, Discs-large; Lgl, lethal giant larvae; APC, adenomatous polyposis coli; MAPK, mitogen-activated protein kinase; cJNK, c-Jun N-terminal kinase; STX4, Syntaxin 4; MPP7, membrane-palmitoylated protein 7.

Figure 3.

Components of the Scribble protein complex in the testis. Scribble, Lgl2 and Dlg1 were detected in the testis, as well as in Sertoli (isolated from 20-day-old rat testes after 4 days in culture with negligible germ cell contamination) and germ cells (isolated from 90-day-old rats after ~12-hr in culture) by RT-PCR. Primers used in PCR were as follows: (i) Scribble: sense, 5'-CTGGCACTGCTCACAGATCT-3 (nucleotides 724-743); antisense, 5'-AGCACCTCAAGATGATTCCG (nucleotides 961-980) (GenBank Accession #:XM_002726943.1); (ii) Lgl2: sense, 5'-TCCACCATCTCGAACACTCG (nucleotides 442-461); antisense, 5'-TGCTGGATGACAACAGCCTG (nucleotides 726-745) (GenBank Accession #:NM_001127549.1); (iii) Dlg1: sense, 5'-GTTGACCTCAGAGCTGCAAG (nucleotides 2000-2019); antisense, 5'-CCACCACTCGTCATCAGAAG (nucleotides 2301-2320) (GenBank Accession #:NM_012788.1); which were co-amplified with (iv) S16: sense, 5'-TCCGCTGCAGTCCGTTCAAGTCTT (nucleotides 177-200); antisense, 5'-GCCAAACTTCTTGGATTCGCAGCG (nucleotides 538-561) (GenBank Accession #:XM_001078234). RT-PCR was performed essentially as earlier described.^{138,139} The cycling parameters for PCR reaction were: denaturation at 94 °C for 1 min, annealing at 57 °C for 2 min, and extension at 72 °C for 3 min, for a total of 21-25 cycles, which were followed by an extension period of 15 min at 72 °C. The identity of the PCR product was confirmed by direct nucleotide sequencing at Genewiz. SC, Sertoli cells; GC, germ cells, bp, base-pair.

Figure 4.

Stage-specific expression of Scribble in the seminiferous epithelium of adult rat testis during the seminiferous epithelial cycle of spermatogenesis. Immunohistochemistry was performed using frozen cross-sections of testes from adult rats and a goat anti-Scribble antibody (Santa Cruz, Cat. # sc-11048) which cross-reacted with Scribble in rats. Using immunoblot analysis and lysates of rat Sertoli cells (SC), this anti-Scribble antibody (at 1:750 dilution for immunoblotting) was found to be specific to Scribble (a). This antibody was used for immunohistochemistry (at 1:50 dilution) (c-g) versus normal goat serum which served as the negative control and shown in (b). Immunoreactive Scribble appears as reddish-brown precipitates (c-g). It is noted that Scribble was localized almost exclusively near the basement membrane in the seminiferous epithelium, consistent with its localization at the BTB (note: this pattern of localization also illustrates its polarized localization) in tubules, most prominently at Stages IV-VIII but it diminished rapidly thereafter to an almost nondetectable level at Stages X-XII but gradually re-appeared from Stage XIII. Bar in b = 80 mm, which applies to c; bar in d = 40 mm, which applies to e-g. Mr, molecular weight. A color version of this figure is available online at www.landesbioscience.com/curie.

Figure 5.

A study by dual-labeled immunofluorescence analysis to assess the colocalization of Scribble with TJ-proteins occludin and ZO-1 at the BTB. In order to further confirm the observations shown in Figure 4 that Scribble is indeed an integrated component of the BTB, dual-labeled immunofluorescence analysis was performed in which Scribble (green fluorescence in b, f) was found to colocalize with either occludin (a TJ-integral membrane protein at the BTB, red fluorescence in a) or ZO-1 (a TJ-associated adaptor protein at the BTB, red fluorescence in e) with the merged images shown in c, d, and g, h, which appear as orange-yellow fluorescence. Cell nuclei were visualized by DAPI staining (d, h). Bar in a = 40 μ m, which applies to b-h. A color version of this figure is available online at www.landesbioscience.com/curie.

Table 1

Components of the Scribble complex in different species

<i>Drosophila</i>		<i>C. elegans</i>		Mammals	
Mr (kDa)		Mr (kDa)		Mr (kDa)	Gene Symbol
Scribble	190	LET-413	75	Scrib1/Crib1/Vartul/hScrib	250*/175 <i>SCRBI</i>
Lgl	127	-		Lgl1	115 <i>LLGL1</i>
				Lgl2	113 <i>LLGL2</i>
				Lgl3/syntaxin-binding protein5	127 <i>LLGL3</i>
				Lgl4/syntaxin-binding protein5-like	130 <i>LLGL4</i>
Dlg	107	Dlg1	107	SAP97/hDlg1	130*/100 <i>DLG1</i>
				PSD93/Chapsyn110	65 <i>DLG2</i>
				SAP102/NE-Dlg	90 <i>DLG3</i>
				SAP90/PSD95	85 <i>DLG4</i>
				LP-Dlg	202 <i>DLG5</i>

* This apparent Mr was obtained by immunoblot analysis based on SDS-PAGE and the corresponding specific antibodies performed in our laboratory using rat testes as lysates.