The Tumor Suppressor SCRIB is a Negative Modulator of the Wnt/β-Catenin Signaling Pathway

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SCRIB is a scaffold protein containing leucine-rich repeats (LRR) and PSD-95/Dlg-A/ZO-1 domains (PDZ) that localizes at the basolateral membranes of polarized epithelial cells. Deregulation of its expression or localization leads to epithelial defects and tumorigenesis in part as a consequence of its repressive role on several signaling pathways including AKT, ERK, and HIPPO. In the present work, a proteomic approach is used to characterize the protein complexes associated to SCRIB and its parologue LANO. Common and specific sets of proteins associated to SCRIB and LANO by MS are identified and an extensive landscape of their associated networks and the first comparative analysis of their respective interactomes are provided. Under proteasome inhibition, it is further found that SCRIB is associated to the β-catenin destruction complex that is central in Wnt/β-catenin signaling, a conserved pathway regulating embryonic development and cancer progression. It is shown that the SCRIB/β-catenin interaction is potentiated upon Wnt3a stimulation and that SCRIB plays a repressing role on Wnt signaling. The data thus provide evidence for the importance of SCRIB in the regulation of the Wnt/β-catenin pathway.

1. Introduction

Epithelial tissues are highly organized structures that rely on cell polarity pathways driven by membrane receptors and scaffolding proteins. SCRIB is a large cytoplasmic scaffold protein present at cell–cell junctions in polarized cells that contains LRR and PDZ domains and is a member of the LAP protein family. The family is composed of DENSIN-180, ERBIN, SCRIB, and LRRC1/LANO (hereafter referred to as LANO). Based on phylogenetic trees, LAP proteins can be split in two branches, one comprising SCRIB and LANO and the second one ERBIN and DENSIN-180.[1]

Loss of SCRIB in Drosophila and mammals leads to cell polarity defects and cancer development.[2,3] In normal epithelial cells, SCRIB is recruited at the plasma membrane through E-cadherin engagement[4] and negatively regulates a set of signaling pathways including AKT,[5] ERK,[6] and HIPPO[7] pathways. SCRIB expression has been shown to regulate cell proliferation, as demonstrated by its tumor suppressor role in mouse mutants.[8,9] It can also associate with oncogenic viral proteins such as human papilloma[10] or T lymphotropic viruses,[11] which promote its degradation or mislocalization. Furthermore, loss of SCRIB expression in conjunction with oncogenic activation of H-Ras[12,13] or c-Myc[9] enhances cancer cell proliferation.

Epithelial organization is important for tissue homeostasis and relies on the self-renewal versus differentiation of stem cells, which is directed by extrinsic short-ranged signals that emanate from the local environment. Wnt signaling is required to control cell differentiation and proliferation.[14] In cancer cells, Wnt signaling is often upregulated and promotes cancer progression by enhancing cell proliferation.[15] At the molecular level, in the absence of Wnt, cytoplasmic β-catenin is constantly ubiquitinated and degraded by the proteasome. This process is governed by a molecular machinery called the β-catenin destruction complex. The destruction complex is composed of the scaffolding proteins Axin, APC, Dvl, Amer1, and the protein kinases CK1α and GSK3α/β, and functions by catalyzing the serine/threonine phosphorylation of a conserved region at the N-terminus of β-catenin. This event promotes the recruitment of the β-TRCP1 E3-ubiquitin ligase and therefore leads to β-catenin proteasome-mediated degradation. The destruction complex is regulated by the secreted glycoprotein Wnt, which binds to a plasma membrane heterodimer including a Frizzled (FZD) family member.
and one of the Wnt co-receptors, LRP5 or LRP6 (LRP5/6). Wnt binding to the FZD-LRP5/6 complex results in the successive recruitment of Dishevelled and the Axin-GSK3 complex. GSK3 in turn phosphorylates the C-terminus domain of LRP5/6. Phosphorylated LRP5/6 has high affinity for Axin, which in turn leads to accumulation of β-catenin and its translocation into the nucleus, where it acts as a transcriptional co-activator by binding to and modulating the activity of the Tcf/LeF family of DNA-binding proteins. In the absence of Wnts, β-catenin is constitutively targeted for proteolysis impairing transcriptional events. In organized epithelial sheets, Wnt signaling is low but the contribution of scaffold proteins to the inhibition of the Wnt/β-catenin remains unclear and might be due to the inhibitory role of adherens junctions and apical-basal polarity complexes.

Little is known about the interactomes of SCRIB and LANO that consists at the moment of 67 and 30 partners, respectively, with only nine common partners (BioGRID, version 3.5). Our study uncovers the proteome associated with SCRIB and LANO and provides, for the first time, a deep comparative analysis of protein interactomes associated to these paralogues. We also identified a protein complex associated with SCRIB, which is stabilized by the inhibition of the proteasome, and further characterized SCRIB as a negative modulator of the Wnt/β-catenin signaling.

2. Experimental Section

2.1. Plasmid Constructs and Reagents

The cDNA encoding human SCRIB and LANO were cloned into pIRES-puro-FLAG, plasmids. Antibodies purchased were mouse anti-FLAG M2 (Sigma), anti-β-catenin (Santa Cruz), anti-SCRIB (Santa Cruz for immunoprecipitation or Cellsignaling for Western Blot detection), and anti-LRR (in-house generated, see ref. [17]). The following sequences of siRNA were used to target SCRIB: SCRIB (#01) GACCGCGUCCUCUCUAUUA, SCRIB (#02) GGACGACGAGGGCGAUAUUC.

2.2. Tissue Culture and Transfection

HEK293T cells were grown in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and penicillin/streptomycin (BioShop) in a 37 °C humidified incubator with 5% CO₂. Stable cell lines were generated either by calcium phosphate transfection or polyethyleneimine (PEI) transfection and selected using puromycin selection during 30 days to generate cell lines expressing stably the desired constructs. Stable expression is confirmed by western blot analysis.

2.3. Immunopurification

A total of 5 × 10⁶ HEK293T cells expressing either FLAG-SCRIB or FLAG-LANO were used for affinity purification procedure. Briefly, cells were lysed and solubilized in tandem affinity purification (TAP) lysis buffer (0.1% Igepal CA 630, 10% glycerol, 50 mM Hepes-NaOH; pH 8.0, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 10 mM NaF, 0.25 mM NaVO3, 50 mM β-glycerophosphate, and protease inhibitor cocktail (Calbiochem). After 30 min centrifugation at 40 000 × g (18 000 rpm in a Beckman JA20 rotor), the soluble fraction (40 mg of proteins) was incubated overnight at 4 °C with anti-FLAG M2 agarose beads (Sigma). Beads were washed with 1/100 (v/v) of TAP lysis buffer. Beads elution was performed using Laemmli buffer.

2.4. Affinity Purification, Immunoprecipitation, Western Blot, and Quantification

Cells were lysed with the TAP lysis buffer and incubated at 4 °C for 1 h to solubilize proteins. Affinity purification and immunoprecipitations were performed using anti-FLAG-M2 beads (Sigma) for 3 h at 4 °C or using 1 µg of anti-SCRIB (Santa Cruz) and 20 µL of protein A beds for 3 h at 4 °C. After extensive washes with lysis buffer, proteins were eluted with 2X Laemmli sample buffer and heated at 95 °C for 5 min in the presence of β-mercaptoethanol (Sigma). Whole cell lysates (50 µg) or purified protein samples were resolved by SDS-PAGE and transferred onto Biotrace NT Nitrocellulose Transfer Membranes (GE Healthcare). Western blotting was performed with antibodies as indicated in the figure legends (anti-FLAG M2 (Sigma) at 1:2000 dilution, anti-β-catenin (Santa Cruz) 1:1000 dilution, anti-SCRIB (Cell signaling) 1:1000 dilution, anti-LRR at 1:1000 dilution, followed by chemiluminescent detection using appropriate horseradish peroxidase (HRP)-conjugated antibodies and the ECL (GE Healthcare) reagent. Western blot quantification was performed using ImageJ software.

2.5. Proteasome Inhibitor Treatment

Depending of the experiments, cells were treated with 1 µM (Figures 2 and 3) or 10 µM MG132 (Sigma) (Figure 3) for 16 or 4 h, respectively, to inhibit proteasome functions.

Significance Statement

Epithelial cell polarity is organized and maintained by a set of PDZ domain containing proteins that includes SCRIB, a cytoplasmic scaffold with tumor suppressing functions. SCRIB belongs to the LRR and PDZ protein family (LAP) that also comprises LANO, a parologue lacking PDZ domains. We set up an unbiased approach to identify the protein complexes associated to SCRIB and LANO by high-resolution MS and provide their first in-depth comparative interactomes. Commonalities and differences of partners were found and future studies will benefit from this resource to further study the functions of these cell polarity components involved in cancer. We also demonstrated that SCRIB is physically and functionally associated to the Wnt/β-catenin complex, and acts as a negative regulator of Wnt/β-catenin as previously shown for LANO. The SCRIB-LANO branch of the LAP family is thus not only characterized by sequence and interactome commonalities but also by common signaling properties that may synergize in cancer development.
2.6. Production of Wnt3a Conditioned Medium

Mouse L cells expressing Wnt3a (CRL-2647) were cultured until reaching 90% confluency. Medium was then collected and refreshed every 2 days for a total of 6 days. Media from different days were assayed by TopFlash assays to determine the fractions with maximal activity and subsequently used for Wnt stimulation experiments. Conditioned media from parental mouse L cells not producing Wnt3a (CRL-2648) were also collected and used as controls. Each production of condition media batch was quality controlled for Wnt response using reporter luciferase assay.

2.7. TopFlash Reporter Assays

Lentivirus containing the TopFlash β-catenin-dependent luciferase reporter (firefly luciferase) and Renilla luciferase were produced and used to establish stable HEK293T and MCF10A Wnt-reporter lines. A total of 25,000 cells were seeded on 24-well plates, followed by DNA transfection with Lipofectamine 2000 and/or reverse transfection with Lipofectamine RNAiMax for siRNA experiments. For experiments involving Wnt stimulation, the medium was replaced 24 h after transfection with a 1:1 mix of fresh DMEM-Wnt3a or DMEM-control conditioned medium. The cells were then assayed 16 h after stimulation in accordance with the dual luciferase assay protocol (Promega) using a plate reader (Berthold LB960).

2.8. Immunocytochemistry and Confocal Microscopy

HEK293T cells were grown on poly-D-lysine (Sigma) treated coverslip and transfected as described in the figure legends. Forty-eight hours post-transfection, cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS and the nonspecific sites were saturated with a 3% BSA (EuroClone) solution in PBS. Samples were immuno-stained with anti-SCRIB (Santa Cruz) or anti-β-catenin (Santa Cruz) antibodies or Phalloidin (Thermo) in 3% BSA. Cells were subsequently labeled with secondary (Thermo) goat anti-goat or mouse anti-body conjugated to Alexa Fluor 488 and Alexa Fluor 594, respectively. Coverslips were mounted and sealed onto slides using ProlongGold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) mounting media (Thermo). Cells were then visualized and images were acquired with a Carl Zeiss LSM880 confocal microscope using a Plan-Apochromat 63× oil immersion objective. Lasers at 488, 543, and 633 nm were triggered independently using the multi-track function of LSM880. Uncompressed images were processed using Zeiss LSM Image browser version ZEN.

2.9. MS Analysis

Proteins precipitated by anti-FLAG immunopurification were loaded on acrylamide gels to stack proteins in a single band that was stained and cut from the gel. Gel pieces were submitted to an in-gel trypsin digestion. Peptides were collected, dried-down and concatenated with 0.1% trifluoroacetic acid in 4% acetonitrile, and analyzed by LC–MS/MS using a LTQ-Orbitrap Velos Mass Spectrometer (Thermo Electron, Bremen, Germany) both online with a nanoRSiLC Ultimate 3000 chromatography system (Dionex, Sunnyvale, CA). Peptides, 16% of the purified samples, were separated using a two steps linear gradient (4-45% acetonitrile/H₂O: 0.1 % formic acid for 120 min (see Supporting Information Materials and Methods for detailed methods).

2.10. Protein Identification and Quantification

Three biological replicates were used for label-free relative quantification. Each replicate was further injected thrice in the system. Briefly, relative intensity-based label-free quantification (LFQ) and intensity-based absolute quantification (iBAQ) intensity were processed using the freely available MaxQuant computational proteomics platform, version 1.6.2.1.[19–21] Spectra were searched against the human database subset of the Swiss-Prot database (date 18/09/19, 20 394 entries) supplemented with a set of frequently observed contaminants and using mainly default parameters. The false discovery rate (FDR) at the peptide and protein levels was set to 1%. The statistical analysis was done with Perseus program (version 1.6.1.3).[22] To determine whether a given detected protein was specifically differential, a two-sample t-test was done using permutation-based FDR controller at 0.01 and employing 250 permutations. The differential proteomics analysis was carried out on identified proteins after removal of proteins only identified with modified peptides, peptides shared with other proteins, proteins from contaminant database, and proteins that are present in 100% in at least one condition. Alternatively, the iBAQ were uploaded from the proteinGroups.txt file and transformed by a base logarithm 2 to obtain a normal distribution. Thus, iBAQ intensities were roughly proportional to the molar quantities of the proteins.

2.11. Proteome Analysis

Gene ontology (GO) and enrichment analyses were performed using the database for annotation, visualization, and integrated discovery (DAVID 6.8)[23] using the full dataset as background. Interaction network were generated using STRING’s website (version 11.0) at high confidence (0.7) and network is clustered using a Markov Clustering algorithm “MCL inflation parameter = 3” (https://string-db.org)[24,25] BioGRID version 3.5.171 was used to extract known SCRIB and LANO interactors.[26,27] The MS proteomics data, including search results, were deposited to the ProteomeXchange Consortium (www.proteomexchange.org) via the PRIDE[28] partner repository with the dataset identifier PXD013636 and PXD013647.

3. Results

3.1. Protein Landscapes Associated to LANO and SCRIB

SCRIB and LANO are paralogue genes that encode proteins sharing 64.5% identity and 85.3% sequence similarity in their 500 first amino acids, which include the Leucine rich repeat domains (LRR) as highlighted using a pairwise sequence
The binding motif has been found in the SCRIB sequence whereas LANO is composed of 524 amino-acids. Four PDZ domains have been identified in SCRIB sequence whereas LANO does not have PDZ domains. A PDZ binding motif (~TSV) is located at the carboxy-terminus end of LANO and no PDZ binding motif has been found in the SCRIB sequence.[1] To further study their functions and highlight possible differences and commonalities in term of partners, we used an unbiased proteomic approach to characterize their associated protein complexes. We first generated stable HEK293T cell lines expressing either FLAG-SCRIB or FLAG-LANO. To identify binding partners of SCRIB and LANO, we used a variation of a previously described methodology[29] based on LC–MS/MS analysis of FLAG immunoprecipitates from HEK293T cell extracts expressing either FLAG-SCRIB or FLAG-LANO, or FLAG alone. The median Pearson correlation across replicates were 0.86, 0.82, and 0.92 for control, LANO, and SCRIB conditions, respectively. Proteins identified from HEK293T cells expressing FLAG alone were compared to proteins associated to either FLAG-SCRIB or FLAG-LANO. Overall 3.5% of the 390 proteins found associated with SCRIB were already known as SCRIB partners in BioGRID, among them a subset of proteins previously characterized by our laboratory and others including ARGHEF7,[30] PAK,[31] and SHOC2,[32] validating our approach (Figure 1B; Table S1, Supporting Information). Similarly, we identified 474 proteins associated with LANO (Figure 1C; Table S1, Supporting Information). Of these, 2% were known LANO interacting partners in BioGRID (Figure 1C; Table S2, Supporting Information). We found that SCRIB was present in the LANO protein complex and conversely that LANO was present in the SCRIB protein complex. We validated these results by conducting co-immunoprecipitation assays followed by western blot analysis. We used stable cell lines expressing either FLAG-SCRIB or FLAG-LANO. After cell lysis and immunoprecipitation using α-FLAG antibody, we monitored the presence of LANO and SCRIB using a specific antibody able to recognize the LRR domains of the two paralogues. We found that LANO co-immunoprecipitates with SCRIB confirming our MS results (Figure 1D). We next analyzed our data set to discriminate common and specific proteins bound to either SCRIB or LANO (Figure 1E; Table S3, Supporting Information). Note that 30% proteins are common to SCRIB and LANO whereas 275 are specifically associated to LANO and 191 specifically associated to SCRIB (Figure 1E; Table S4, Supporting Information). Several PDZ domain containing proteins were found in the SCRIB and LANO interactomes. Six proteins (DVL2, PDLIM7, TJP2, ARHGA21, MAGI1, and MPP2) were common to the two paralogues whereas four were specific to LANO (DLG1, CASK, MPP7, LIN7C, and and SNX27) and five to SCRIB (PDZ8, GOPC, SIPA1L1, SIPA1L2, SIPA1L3, and PTPN13) (Figure 1C,E; Tables S1 and S2, Supporting Information). We confirmed in particular our previous findings showing that DLG1 is a specific LANO partner.[33] Given the reciprocal pull-down of SCRIB and LANO, we generated a volcano plot showing the respective abundance of the associated proteins to each paralogue (Figure S1, Supporting Information). We identified that 191 proteins (green) are mostly associated to SCRIB and 275 proteins (blue) are mostly associated to LANO whereas 199 proteins (grey) are found equally within SCRIB and LANO associated protein complex (Table S4, Supporting Information). Taken together, we provide the most exhaustive list of proteins associated with either SCRIB or LANO and show that SCRIB and LANO bind to common and different proteins.

3.2. SCRIB is Associated with the β-Catenin Destruction Complex

It has been recently shown that SCRIB acts in the HIPPO pathway by scaffolding β-TRCP1, promoting TAZ degradation at the plasma membrane of epithelial cells.[7] We did not observe the presence of either TAZ or β-TRCP1 among the SCRIB associated proteins. We then asked whether this could be due to protein degradation and thus carried out our proteomic approach after treatment of FLAG-SCRIB expressing HEK293T cells with MG132, a proteasome inhibitor. To identify binding partners of SCRIB under MG132 treatment, we used a similar LC–MS/MS proteomic analysis of FLAG immunoprecipitates from HEK293T cell extracts expressing FLAG-SCRIB. The median Pearson correlations across replicates were 0.92 and 0.93 for DMSO or MG132 treatments, respectively. We compared the list of SCRIB-associated proteins under DMSO or MG132 treatments. We did not observe any variation of SCRIB expression by MS analysis (Figure 2A; Table S5, Supporting Information) or western blot analysis (data not shown) suggesting that SCRIB is a relatively stable protein, poorly sensitive to proteasomal degradation. However, we found that 37 proteins were differentially associated to SCRIB upon inhibition of the proteasome (11 downregulated and 26 upregulated proteins). Among the partners, we identified β-catenin (log2 ratio 7.90 and p-value 3 × 10⁻⁹) as a protein specifically associated with SCRIB under MG132 treatment (Figure 2A). Using DAVID bioinformatic resources,[23] we also observed an enrichment of a subset of proteins associated to SCRIB that are involved in Wnt/β-catenin signaling, in particular members of the β-catenin destruction complex that includes APC, Axin, AMER1, DVL, and GSK3β (Figure 2B). Further analysis using STRING “functional protein association networks”[24] highlighted a protein–protein association network associated to SCRIB (Figure 2C). We thus evidenced a highly connected network of 17 proteins associated to SCRIB that comprises ten proteins involved in Wnt/β-catenin signaling (Figure 2B).

3.3. Wnt3a Stimulation Promotes SCRIB Association with β-Catenin

To confirm the MS data, we conducted immunoprecipitation of endogenous SCRIB using specific antibody, and monitored the presence of endogenous β-catenin by western blot analysis. We observed that SCRIB and β-catenin association was promoted under MG132 treatment at two different time points and at two different concentrations (Figure 3A). We also observed a modification of the migration pattern of β-catenin in polyacrylamide gels, which is typical of ubiquitinated forms, suggesting that SCRIB might promote degradation of β-catenin through its association with the destruction complex (Figure 3A). As the destruction complex is inhibited by Wnt stimulation, we next stimulated cells overnight with Wnt3a conditioned media and conducted immunoprecipitation of endogenous SCRIB and looked at the presence of β-catenin in the SCRIB complex. We observed that
Figure 1. Protein landscapes associated to SCRIB and LANO. A) Domain structure of SCRIB and LANO showing sequence homologies. The percent of homology is given using the LALIGN tool (https://www.ebi.ac.uk/Tools/psa/lalign/). B, C) MS analysis of the protein complexes after FLAG immunopurification of proteins from HEK293T cells expressing FLAG-SCRIB (B) or FLAG-LANO (C). Volcano plots showing differential log2 (iBAQ intensity) levels (x-axis) and –log(p-value) (y-axis) for SCRIB versus control (B) and LANO versus control (C). The full lines are indicative of significant hits obtained at a log2 difference of 2 and a –log(p-value) of 2 and are highlighted in green for FLAG-SCRIB versus control conditions (B), in red for FLAG-LANO versus control conditions (C), and in grey for proteins with nonsignificant changes. D) Immunopurification of FLAG-SCRIB (lane 3) or FLAG-LANO (lane 4) extracted from HEK293T cell lysates using anti-FLAG antibody coupled to sepharose beads allows the identification of a SCRIB (red arrow)-LANO (green arrow) interaction. Expression of FLAG-SCRIB and FLAG-LANO in lysates are shown lane 1 and lane 2, respectively. E) Venn diagram showing number of total proteins (n) or PDZ domain proteins (n (PDZ)) associated to SCRIB and/or LANO. B, C) Known SCRIB and LANO interactors indexed in BioGRID are highlighted in red and PDZ proteins are highlighted in orange.
Figure 2. SCRIB associates to the $\beta$-catenin destruction complex. A) Volcano plots showing differential log2 (LFQ intensity) levels (x-axis) and $-\log(p$-value) (y-axis) for proteins associated to SCRIB under DMSO or MG132 treatments. The dotted lines are indicative of significant hits obtained at a log2
Wnt3a stimulation promotes the association between SCRIB and β-catenin by 1.8-fold (Figure 3B). To further confirm this observation, we treated cells with LiCl, an inhibitor of GSK3β leading to β-catenin stabilization, and assessed endogenous SCRIB/β-catenin co-immunoprecipitation. We observed that the association between SCRIB and β-catenin is potentiated when GSK3β is inhibited, suggesting that this interaction is regulated by activation of Wnt/β-catenin signaling (Figure 3C). We further assessed the colocalization between SCRIB and β-catenin in HEK 293T cells by immunofluorescence. We observed that both proteins colocalize at the plasma membrane with no difference in their subcellular localization following Wnt3a-conditioned media or LiCl treatments (Figure 3D). Taken together, our data suggest that SCRIB is associated with β-catenin under basal conditions. However, this interaction is promoted when β-catenin is stabilized by Wnt3a or LiCl treatments.

3.4. SCRIB is a Negative Regulator of the Wnt/β-Catenin Signaling

Since SCRIB is associated with β-catenin and this interaction is modulated by Wnt3a stimulation, we next asked whether SCRIB can act as a modulator of Wnt/β-catenin signaling. We first screened siRNAs able to downregulate SCRIB in human cells. We identified two independent siRNAs able to decrease SCRIB expression by at least 50% (Figure 4A). HEK293T and MCF10A cell lines stably expressing a β-catenin luciferase reporter and a Renilla luciferase control protein were then transfected with either control or SCRIB siRNAs. In the control transfected MCF10A and HEK293T cells, addition of Wnt3a led to 6- and 65-fold activity of the reporter, respectively (Figure 4B,C) compared to cells treated with control conditioned medium. Depletion of SCRIB led to an increase in Wnt3a-mediated activation by 22- and 167-fold in MCF10A and HEK293T cells, respectively (Figure 4B,C). We therefore conclude that SCRIB acts as a negative regulator of Wnt signaling. It has been previously shown that SCRIB associates to β-catenin through its PDZ domains.[34]

To further investigate the role of SCRIB as a negative regulator of Wnt/β-catenin signaling, we overexpressed full length SCRIB fused to green fluorescent protein (GFP) in HEK293T cells and observed a partial decrease of activity of the luciferase reporter (Figure 4D). However, when we overexpressed a mutant form of SCRIB lacking its PDZ domains (GFP-SCRIBΔPDZ), we did not observe any difference with control conditions, confirming that the PDZ domains of SCRIB are required for its repressive function (Figure 4D). Expression levels of GFP-SCRIB and GFP-SCRIBΔPDZ were controlled by western blot analysis (Figure 4E). Together our data demonstrate the importance of SCRIB as a negative regulator of Wnt/β-catenin signaling, probably through its direct interaction with β-catenin.

4. Discussion

We and others have demonstrated the importance of SCRIB and LANO in cancer development.[3,9,12,35] In this study, we identified large protein complexes associated with these two paralogues and carried out for the first time a direct comparison of their associated protein complexes. Using an established functional proteomic approach, our results show that SCRIB and LANO interact with a common set of 199 proteins. Furthermore, we demonstrate that LANO, which has high sequence homology with SCRIB in its N-terminal region (Figure 1A), interacts with a specific set of 275 proteins that include four PDZ domain-containing proteins (DLG1 CASK, MPP7, and SNX27) and SCRIB with 191 specific partners that include six PDZ containing proteins (PDZ8, GOPC, SIPA1L1, SIPA1L2, SIPA1L3, PTPN13). We further characterized proteins associated with SCRIB in conditions where we inhibited proteasome activity and found that this treatment led to the association between SCRIB and the β-catenin destruction complex. Further analysis revealed that SCRIB is a negative regulator of the Wnt/β-catenin signaling pathway. Our data suggest that SCRIB scaffolds components of the destruction complex and most probably inhibits the membrane pool of β-catenin (Figure 3D). Indeed, downregulation of SCRIB expression promotes an increase of Wnt/β-catenin signaling using reporter assays. In conclusion, our data suggest that SCRIB may control Wnt signaling in epithelial cells.

Our study decipher, for the first time, the protein complexes associated with proteins encoded by the two close paralogues SCRIB and LANO. We demonstrate that SCRIB and LANO can associate since specific LANO peptides have been identified by LC–MS in the SCRIB complex (Figure 1B) and conversely that specific SCRIB peptides were found in the LANO complex (Figure 1C). According to the abundance of LANO and SCRIB in subsequent LC–MS showing no decrease, a carry-over cannot be suspected. In addition, the SCRIB–LANO interaction was further confirmed by co-immunoprecipitation (Figure 1D). Interestingly, we have recently demonstrated that LANO and SCRIB colocalize at the basolateral membranes of epithelial cells.[10] LANO harbors a C-terminal PDZ binding motif that allows its interaction with a subset of PDZ domain-containing proteins including DLG1, SNX27, or SCRIB (Figure 1A).[3] Although contribution of LANO to SCRIB functions or vice-versa has not yet been studied, we show here and in a recent report[36] that both proteins negatively regulate Wnt/β-catenin signaling suggesting overlapping functions. Our extensive analysis of their associated protein complexes will help in the future at a better understanding of their respective role in epithelial cells. Interestingly, SCRIB knock-out mice display morphogenetic defects[37] whereas Lano deficient mice are viable and fertile with no obvious phenotype.[36] These genetic results suggest that the two paralogues contribute differently to murine embryonic development and, combined to the results of our comparative analysis of protein complexes.
**Figure 3.** Wnt3a stimulation promotes SCRIB association with β-catenin. A–C) Immunopurification of endogenous SCRIB and presence of β-catenin in the complex was assessed by western blot analysis. Before lysis, cells were treated with MG132 for the indicated times and concentrations (A). Cells were treated with Ctl- or Wnt3a-condition media for 16 h before lysis (B). Cells were treated with 10 mM LiCl or 10 mM NaCl for 16 h before lysis, quantification represents combine value of three independent experiments (bottom). Statistical analysis was performed using Student’s t-test. Statistical analysis was performed against control (white histogram). *p < 0.05. Data are presented as mean ± SEM. C,D) HEK293T cells were seeded on poly-d-lysine coverslips and treated with 10 mM NaCl, 10 mM LiCl, Ctl-CM, or Wnt3a-CM for 16 h. After fixation, cells were stained with phalloidin (purple) to reveal polymerized actin, anti-SCRIB antibody (green), anti-β-catenin antibody (red), and DAPI (blue). Scale bars 20 μm.

interactomes, that SCRIB and LANO have probably both redundant and nonredundant functions. It would be of great interest in the future to cross scrib and lano deficient mice and assess a possible functional interaction between these genes.

A functional proteomic approach has been used to demonstrate that SCRIB is associated with a specific protein complex involved in Wnt/β-catenin signaling pathway. Our results suggest that SCRIB is a negative modulator of Wnt/β-catenin signaling. It has been recently shown that SCRIB, when localized at the plasma membrane, recruits β-TRCP, an E3-ubiquitin ligase that promotes degradation of TAZ in a proteasome-dependent manner, leading to inhibition of the HIPPO signaling pathway.\(^7\)
Figure 4. SCRIB is a negative regulator of Wnt/β-catenin signaling. A) Two independent siRNAs targeting SCRIB or a control siRNA (non-targeting, NT) were transfected into HEK 293T cells. Decrease of SCRIB expression was confirmed by western blot analysis. B,C) MCF10A (B) or HEK 293T (C) cells stably expressing the TOP Flash luciferase reporter were transfected with the indicated siRNAs and, after 24 h, were treated with either wild-type conditioned media (WT-CM) or Wnt3a conditioned media (Wnt3a-CM) for 16 h. The luciferase reporter activity was measured with a luminometer (mean ± SD; n = 3). D) HEK 293T cells transfected with the indicated constructs were treated with WT-CM or Wnt3a-CM for 16 h. E) Expression of GFP-SCRIB constructs was assessed by western blot analysis. Fold activations are standardized to 1 using the value obtained from the control condition, first white bar from the left representing siRNA N.T. and treated with WT-CM. Statistical analysis was performed using using one-way ANOVA with Tukey post-test. **p < 0.01; ***p < 0.001.
Furthermore, TAZ is also a component of the β-catenin destruction complex that through recruitment of β-TRCP, degrades β-catenin.38 Our initial proteomic approach done under basal conditions (Figure 1B) failed to identify components of HIPPO or Wnt signaling pathways associated to SCRIB. However, by co-immunoprecipitation and western blot analysis of endogenous SCRIB, we could recover a SCRIB/β-catenin complex (Figure 3) but not SCRIB/TAZ complex (data not shown) suggesting that very few β-catenin is associated to SCRIB under basal conditions. Under MG132 treatment, we were able to easily identify by MS β-catenin and members of the β-catenin (but still not TAZ) destruction complex associated with SCRIB demonstrating that, at least in our cellular model systems, the SCRIB/β-catenin destruction complex is predominant and regulated directly or indirectly by proteosomal degradation. We next showed that SCRIB behaves as a negative modulator of this signaling pathway as it does with AKT, ERK, or HIPPO pathways (Figure 4B). It has been recently shown that LANO represses Wnt/β-catenin signaling and promotes the maintenance of breast cancer stem cells.36 It would be interesting to assess a possible functional cooperation between the two paralogues in this setting (Figure 1D).

Finally, β-catenin is a central component of the cadherin cell adhesion complex and plays an essential role in the maintenance of the epithelial structures.39 Studies have shown that SCRIB subcellular localization is altered in colorectal carcinoma and correlates with intracellular accumulation of β-catenin compared to normal adjacent epithelia.40 Furthermore, it has been recently shown that SCRIB is required for β-catenin localization at the plasma membrane in order to maintain the integrity of tight junctions.14 Our findings suggest that deregulation of expression of SCRIB may potentiate Wnt/β-catenin signaling and promotes cancer development.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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