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

Avais M Daulat, Mônica Silveira Wagner, Alexandra Walton, Emilie Baudelet, Stéphane Audebert, Luc Camoin, Jean-Paul Borg

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3 1 **The tumor suppressor SCRIB is a negative modulator of the Wnt/ β -catenin signaling**
4
5 2 **pathway**

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8 3 Avais M. Daulat^{1,§}, Mônica Silveira Wagner^{1,§}, Alexandra Walton¹, Emilie Baudelet²,
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10 4 Stéphane Audebert², Luc Camoin^{2,#,*}, Jean-Paul Borg^{1,2,#,*}

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22 9 ¹Centre de Recherche en Cancérologie de Marseille, Equipe labellisée Ligue ‘Cell polarity,
23
24 10 cell signaling and cancer’, Aix-Marseille Université, Inserm, CNRS, Institut Paoli Calmettes,
25
26 11 13009 Marseille, France

27
28
29 12 ²Centre de Recherche en Cancérologie de Marseille, Marseille Proteomics, Aix-Marseille
30
31 13 Université, Inserm, CNRS, Institut Paoli Calmettes, 13009 Marseille, France

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33
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37
38 16 * To whom correspondence should be addressed: luc.camoin@inserm.fr/[jean-](mailto:jean-paul.borg@inserm.fr)
39
40 17 paul.borg@inserm.fr/ Phone 33-4-8697-7201, Fax 33-4-8697-7499

41
42 18 § Co-first authors

43
44 19 # Co-lead authors

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47
48
49 21 **Keywords:** SCRIB, LANO, tumor suppressor, cancer, Wnt

1
2
3 **26 Abstract**
4

5 27 SCRIB is a scaffold protein containing LRR and PDZ domains which localizes at the
6
7 28 basolateral membranes of polarized epithelial cells. Deregulation of its expression or
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9
10 29 localization leads to epithelial defects and tumorigenesis in part as a consequence of its
11
12 30 repressive role on several signaling pathways including AKT, ERK and HIPPO. In the present
13
14 31 work, we used a proteomic approach to characterize the protein complexes associated to
15
16 32 SCRIB and its paralogue LANO. We identified common and specific sets of proteins
17
18 33 associated to SCRIB and LANO by mass spectrometry and provide an extensive landscape of
19
20 34 their associated networks, and the first comparative analysis of their respective interactomes.
21
22 35 Under proteasome inhibition, we further found that SCRIB was associated to the β -catenin
23
24 36 destruction complex which is central in Wnt/ β -catenin signaling, a conserved pathway
25
26 37 regulating embryonic development and cancer progression. We show that the SCRIB/ β -
27
28 38 catenin interaction is potentiated upon Wnt3a stimulation and that SCRIB plays a repressing
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31 39 role on Wnt signaling. Our data thus provide evidence for the importance of SCRIB in the
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35 40 regulation of the Wnt/ β -catenin pathway.
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42 **Statement of significance of the study**

43 Epithelial cell polarity is organized and maintained by a set of PDZ domain containing
44 proteins which includes SCRIB, a cytoplasmic scaffold with tumor suppressing functions.

45 SCRIB belongs to the LAP protein family which also comprises LANO, a paralogue lacking
46 PDZ domains. We set up an unbiased approach to identify the protein complexes associated to

47 SCRIB and LANO by high-resolution mass spectrometry and provide their first in-depth
48 comparative interactomes. Commonalities and differences of partners were found and future

49 studies will benefit from this resource to further study the functions of these cell polarity
50 components involved in cancer. We also demonstrated that SCRIB is physically and

51 functionally associated to the Wnt/ β -catenin complex, and acts as a negative regulator of
52 Wnt/ β -catenin as previously shown for LANO. The SCRIB-LANO branch of the LAP family

53 is thus not only characterized by sequence and interactome commonalities but also by
54 common signaling properties that may synergize in cancer development.

55

56 **Introduction**

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

64 Loss of SCRIB in *Drosophila* and mammals leads to cell polarity defects and cancer
65 development [2],[3]. In normal epithelial cells, SCRIB is recruited at the plasma membrane
66 through E-cadherin engagement [4] and negatively regulates a set of signaling pathways
67 including AKT [5], ERK [6] and HIPPO [7] pathways. SCRIB expression has been shown to
68 regulate cell proliferation, as demonstrated by its tumor suppressor role in mouse mutants [8,
69 9]. It can also associate with oncogenic viral proteins such as human papilloma [10] or T
70 lymphotropic viruses [11, 12] which promote its degradation or mislocalization. Furthermore,
71 loss of SCRIB expression in conjunction with oncogenic activation of H-Ras [13] [14] or c-
72 Myc [9] enhances cancer cell proliferation.

73 Epithelial organization is important for tissue homeostasis and relies on the self-renewal
74 versus differentiation of stem cells which is directed by extrinsic short-ranged signals that
75 emanate from the local environment. Wnt signaling is required to control cell differentiation
76 and proliferation [15]. In cancer cells, Wnt signaling is often up-regulated and promotes
77 cancer progression by enhancing cell proliferation [16]. At the molecular level, in the absence
78 of Wnt, cytoplasmic β -catenin is constantly ubiquitinated and degraded by the proteasome.
79 This process is governed by a molecular machinery called the β -catenin destruction complex.
80 The destruction complex is composed of the scaffolding proteins Axin, APC, Dvl, Amer1 and

1
2
3 81 the protein kinases CK1 α and GSK3 α/β , and functions by catalyzing the serine/threonine
4
5 82 phosphorylation of a conserved region at the N-terminus of β -catenin. This event promotes
6
7 83 the recruitment of the β -TRCP1 E3-ubiquitin ligase and therefore leads to β -catenin
8
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10 84 proteasome-mediated degradation. The destruction complex is regulated by the secreted
11
12 85 glycoprotein Wnt which binds to a plasma membrane heterodimer including a Frizzled (FZD)
13
14 86 family member and one of the Wnt co-receptors, LRP5 or LRP6 (LRP5/6). Wnt binding to the
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16
17 87 FZD-LRP5/6 complex results in the successive recruitment of Dishevelled and the Axin-
18
19 88 GSK3 complex. GSK3 in turn phosphorylates the C-terminus domain of LRP5/6.
20
21 89 Phosphorylated LRP5/6 has high affinity for Axin which in turn leads to accumulation of β -
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24 90 catenin and its translocation into the nucleus, where it acts as a transcriptional co-activator by
25
26 91 binding to and modulating the activity of the Tcf/Lef family of DNA-binding proteins. In the
27
28 92 absence of Wnts, β -catenin is constitutively targeted for proteolysis impairing transcriptional
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31 93 events [17]. In organized epithelial sheets, Wnt signaling is low but the contribution of
32
33 94 scaffold proteins to the inhibition of the Wnt/ β -catenin remains unclear and might be due to
34
35 95 the inhibitory role of adherens junctions and apical-basal polarity complexes [18].
36
37
38 96 Very few is known about the interactomes of SCRIB and LANO which consists at the
39
40 97 moment of 67 and 30 protein partners, respectively, with only 9 common partners (BioGRID,
41
42 98 version 3.5). Our study uncovers the proteomes associated with SCRIB and LANO and
43
44 99 provides, for the first time, a deep comparative analysis of protein interactomes associated to
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47 100 these paralogues. We also identified a protein complex associated with SCRIB, which is
48
49 101 stabilized by the inhibition of the proteasome, and further characterized SCRIB act as
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51 102 negative modulator of Wnt/ β -catenin signaling.
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105 **Materials and Methods**

106 **Plasmid constructs and reagents**

107 The cDNA encoding human SCRIB and IANO were cloned into pIRES-puro-FLAG,
108 plasmids [19]. Antibodies purchased were mouse anti-FLAG M2 (Sigma), anti- β -catenin
109 (Santa Cruz), anti-SCRIB (Santa Cruz). The following sequences of siRNA were used to
110 target SCRIB: SCRIB (#05) GACCGCGUCCUCUCUAUUA, SCRIB (#06)
111 GGACGACGAGGGCGAUUUC.

113 **Tissue culture and transfection**

114 HEK293T cells were grown in Dulbecco's modified Eagle medium (DMEM; Sigma)
115 supplemented with 10% fetal bovine serum (FBS; Invitrogen) and penicillin/streptomycin
116 (Bioshop) in a 37°C humidified incubator with 5% CO₂. Stable cell lines were generated
117 either by calcium phosphate transfection or polyethylenimine (PEI) transfection.

119 **Immunopurification**

120 5×10^7 HEK293T cells expressing either FLAG-SCRIB or FLAG-LANO were used for
121 affinity purification procedure. Briefly, cells were lysed and solubilized in TAP lysis buffer
122 (0.1% Igepal CA 630, 10% glycerol, 50mM HEPES-NaOH; pH 8.0, 150mM NaCl, 2mM
123 EDTA, 2mM DTT, 10mM NaF, 0.25mM NaOVO₃, 50mM β -glycerophosphate, and protease
124 inhibitor cocktail (Calbiochem). After 30 min centrifugation at 40,000xg (18,000 rpm in a
125 Beckman JA20 rotor), the soluble fraction was incubated overnight at 4°C with anti-FLAG
126 M2 agarose beads (Sigma). Beads were washed with 100 volumes of TAP lysis buffer. Beads
127 elution was performed using Laemmli buffer.

129 **Affinity purification, immunoprecipitation and western blot**

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2
3 130 48 hour post-transfection, cells were lysed with the TAP lysis buffer and incubated at 4°C for
4
5 131 1 hour to solubilize proteins. Affinity purification and immunoprecipitations were performed
6
7 132 using anti-FLAG-M2 beads (Sigma) for 3 hours at 4°C or using 1µg of anti-SCRIB (Santa
8
9 133 Cruz) and 20µL of protein A beds for 3 hours at 4°C. After extensive washes with lysis
10
11 134 buffer, proteins were eluted with 2x Laemmli sample buffer and heated at 95°C for 5 min in
12
13 135 the presence of β-mercaptoethanol (Sigma). Whole cell lysates or purified protein samples
14
15 136 were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto
16
17 137 Biotrace NT Nitrocellulose Transfer Membranes (GE Healthcare). Western blotting was
18
19 138 performed with antibodies as indicated in the figure legends, followed by chemiluminescent
20
21 139 detection using appropriate HRP-conjugated antibodies and the ECL (GE Healthcare) reagent.
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28 141 **MG132 treatment**

29
30 142 Depending of the experiments, cells were treated with 1 µM or 10 µM MG132 for 16 h or 4 h
31
32 143 respectively to inhibit proteasome functions.
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37 145 **Production of Wnt3a conditioned medium**

38
39 146 Mouse L cells expressing Wnt3a (CRL-2647) were cultured until reaching 90% confluency.
40
41 147 Medium was then collected and refreshed every 2 days for a total of 6 days. Media from
42
43 148 different days were assayed by TopFlash assays to determine the fractions with maximal
44
45 149 activity and subsequently used for Wnt stimulation experiments. Conditioned media from
46
47 150 parental mouse L cells not producing Wnt3a (CRL-2648) were also collected and used as
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49 151 controls.
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56 153 **TopFlash reporter assays.**

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3 154 Lentivirus containing the TopFlash β -catenin-dependent luciferase reporter (firefly luciferase)
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5 155 and *Renilla* luciferase were produced and used to establish stable HEK293T and MCF10A
6
7 156 Wnt-reporter lines. Cells were seeded on 24-well plates, followed by cDNA transfection with
8
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10 157 Lipofectamine 2000 and/or reverse transfection with Lipofectamine RNAiMax for siRNA
11
12 158 experiments. For experiments involving Wnt stimulation, the medium was replaced with a 1:1
13
14 159 mix of fresh DMEM-Wnt3a or DMEM-control conditioned medium. The cells were then
15
16
17 160 assayed 24 h after stimulation in accordance with the dual luciferase assay protocol (Promega)
18
19 161 using a plate reader (Berthold LB960).
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23 24 163 **Immunocytochemistry and confocal microscopy**

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26 164 HEK293T cells were grown on poly-D-Lysine (Sigma) treated coverslip and transfected as
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28 165 described in the figure legends. 48 hour post-transfection, cells were fixed in 4%
29
30 166 paraformaldehyde, permeabilized with 0.2% Triton X-100 in phosphate buffered saline (PBS)
31
32
33 167 and the non-specific sites were saturated with a 3% BSA (Euromedex) solution in PBS.
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35 168 Samples were immuno-stained with anti-SCRIB (Santa Cruz) or anti- β -catenin (Santa Cruz)
36
37 169 antibodies or Phalloidin (Thermo) in 3% BSA. Cells were subsequently labeled with
38
39
40 170 secondary (Thermo) goat anti-goat or mouse antibodies conjugated to Alexa Fluor 488 and
41
42 171 Alexa Fluor 594 respectively. Coverslips were mounted and sealed onto slides using
43
44 172 ProlongGold antifade reagent with DAPI mounting media (Thermo). Cells were then
45
46
47 173 visualized and images acquired with a Carl Zeiss LSM880 confocal microscope using a Plan-
48
49 174 Apochromat 63X oil immersion objective. Lasers at 488nm, 543nm and 633nm were
50
51 175 triggered independently using the multi-track function of LSM880. Uncompressed images
52
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54 176 were processed using Zeiss LSM Image browser version ZEN.

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57 58 59 178 **Mass spectrometry analysis.**

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3 179 Protein extract were loaded on acrylamide gels to stack proteins in a single band that was
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5 180 stained and cut from the gel. Gels pieces were submitted to an in-gel trypsin digestion¹.
6
7 181 Peptides were collected, dried-down and reconstituted with 0.1% trifluoroacetic acid in 4%
8
9
10 182 acetonitrile and analyzed by liquid chromatography (LC)-tandem mass spectrometry
11
12 183 (MS/MS) using a LTQ-Orbitrap Velos Mass Spectrometer (Thermo Electron, Bremen,
13
14 184 Germany) or using an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Electron,
15
16 185 Bremen, Germany) both online with a nanoRSLC Ultimate 3000 chromatography system
17
18 186 (Dionex, Sunnyvale, CA). Peptides were separated using a two steps linear gradient (4-45%
19
20 187 acetonitrile/H₂O; 0.1 % formic acid for 120 min (see supplementary Materials and Methods
21
22 188 for detailed methods).
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28 190 **Protein identification and quantification.**

29
30 191 Relative intensity-based label-free quantification (LFQ) and iBAQ intensity were processed
31
32 192 using the freely available MaxQuant computational proteomics platform, version 1.6.2.1. [20-
33
34 193 22] Spectra were searched against the human database subset of the Swiss-Prot database (date
35
36 194 18/09/19, 20394 entries) supplemented with a set of frequently observed contaminants and
37
38 195 using mainly default parameters. The false discovery rate (FDR) at the peptide and protein
39
40 196 levels were set to 1%. The statistical analysis was done with Perseus program (version
41
42 197 1.6.1.3) [23]. To determine whether a given detected protein was specifically differential a
43
44 198 two-sample t-test were done using permutation based FDR-controlled at 0.01 and employing
45
46 199 250 permutations. The differential proteomics analysis was carried out on identified proteins
47
48 200 after removal of proteins only identified with modified peptides, peptides shared with other
49
50 201 proteins, proteins from contaminant database and proteins which are present in 100% in at
51
52 202 least one condition. Alternatively, The iBAQ (Intensity Based Absolute Quantification) were
53
54 203 uploaded from the proteinGroups.txt file and transformed by a base logarithm 2 to obtain a
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3 204 normal distribution. Thus, iBAQ intensities are roughly proportional to the molar quantities of
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5 205 the proteins.
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10 207 **Proteome analysis**

12 208 Gene ontology (GO) and enrichment analyses were performed using the database for
14 209 annotation, visualization, and integrated discovery (DAVID 6.8) [24, 25] using the full dataset
16 210 as background. Interaction network were generated using STRING's website (version 11.0) at
18 211 high confidence (0.7) and network is clustered to a specified "MCL inflation parameter = 3"
20 212 [26, 27]. BioGRID version 3.5.171 was used to extract known SCRIB and LANO interactors
22 213 [28] [29] [28]. The mass spectrometry proteomics data, including search results, have been
24 214 deposited to the ProteomeXchange Consortium (www.proteomexchange.org) via the
26 215 PRIDE[30] partner repository with the dataset identifier PXD013636 and PXD013647.

30 216 They can be accessed for reviewing purposes with the following login details :

32 217 Project accession: PXD013636; Username: reviewer40726@ebi.ac.uk; Password: qMsZ4H9A

34 218 Project accession: PXD013647; Username: reviewer18814@ebi.ac.uk; Password: y93bdeTJ

36 219

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221 **Results**

222 **Protein landscapes associated to LANO and SCRIB**

223 *SCRIB* and *LANO* are paralogue genes that encode proteins sharing 72% sequence homology
224 (**Fig 1A**). To further study their functions and highlight possible differences and
225 commonalities in term of partners, we used an unbiased proteomic approach to characterize
226 their associated protein complexes. We first generated stable HEK293T cell lines expressing
227 either FLAG-SCRIB or FLAG-LANO. To identify binding partners of SCRIB and LANO, we
228 used a variation of a previously described methodology [31] based on LC-MS/MS analysis of
229 FLAG immunoprecipitates from HEK293T cell extracts expressing either FLAG-SCRIB or
230 FLAG-LANO or FLAG alone. Proteins identified from HEK293T cells expressing FLAG
231 alone were compared to proteins associated to either FLAG-SCRIB or FLAG-LANO. 3.5%
232 of the 390 proteins found associated with SCRIB were already known as SCRIB partners in
233 BioGRID, among them a subset of proteins previously characterized by our laboratory and
234 others including ARGHEF7 [32], PAK [33] and SHOC2 [34], validating our approach (**Fig.**
235 **1B** and **Table S1**). Similarly, we identified 474 proteins associated with LANO (**Fig.1C** and
236 **Table S1**). Of these, 2% were known LANO interacting partners in BioGRID (**Fig. 1C** and
237 **Table S2**). We found that SCRIB was present in the LANO protein complex and conversely
238 that LANO was present in the SCRIB protein complex. We validated these results by
239 conducting co-immunoprecipitation assays followed by western blot analysis. We used stable
240 cell lines expressing either FLAG-SCRIB or FLAG-LANO. After cell lysis and
241 immunoprecipitation using α -FLAG antibody, we monitored the presence of LANO and
242 SCRIB using a specific antibody able to recognize the LRR domains of the two paralogs. We
243 found that LANO co-immunoprecipitates with SCRIB confirming our MS results (**Fig. 1D**).
244 We next analyzed our data set to discriminate common and specific proteins bound to either
245 SCRIB or LANO (**Fig. 1E** and **Table S3**). 30% proteins are common to SCRIB and LANO

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3 246 whereas 275 are specifically associated to LANO and 191 specifically associated to SCRIB
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5 247 (**Fig. 1E** and **Table S4**). Several PDZ domain containing proteins were found in the SCRIB
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7 248 and LANO interactomes. 6 proteins (DVL2, PDLIM7, TJP2, ARHGAP21, MAGI1, MPP2)
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9
10 249 were common to the two paralogues whereas 4 were specific to LANO (DLG1, CASK,
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12 250 MPP7, LIN7C and and SNX27) and 5 to SCRIB (PDZ8, GOPC, SIPA1L1, SIPA1L2,
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14 251 SIPA1L3, PTPN13) (**Fig. 1C** and **Fig. 1E** and **Table S1** and **Table S2**). We confirmed in
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16
17 252 particular our previous findings showing that DLG1 is a specific LANO partner [35].
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19 253 Taken together, we provide the most exhaustive list of proteins associated with either SCRIB
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21 254 or LANO and show that SCRIB and LANO bind to common and different proteins.
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25 256 **SCRIB is associated with the β -catenin destruction complex**

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27
28 257 It has been recently shown that SCRIB acts in the HIPPO pathway by scaffolding β -TRCP1,
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30 258 promoting TAZ degradation at the plasma membrane of epithelial cells [7]. We did not
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33 259 observe the presence of either TAZ or β -TRCP1 among the SCRIB associated proteins. We
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35
36 260 then asked whether this could be due to protein degradation and thus carried out our
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38 261 proteomic approach after treatment of FLAG-SCRIB expressing HEK293T cells with
39
40 262 MG132, a proteasome inhibitor. We compared the list of SCRIB-associated proteins under
41
42
43 263 DMSO or MG132 treatments. We did not observe any variation of SCRIB expression by MS
44
45 264 analysis (**Fig.2A** and **Table S5**) or western blot analysis (data not shown) suggesting that
46
47 265 SCRIB is a relatively stable protein, poorly sensitive to proteasomal degradation. However,
48
49
50 266 we found that 37 proteins were differentially associated to SCRIB upon inhibition of the
51
52 267 proteasome (11 down-regulated and 26 up-regulated proteins). Among the partners, we
53
54 268 identified β -catenin as a protein specifically associated with SCRIB under MG132 treatment
55
56 269 (**Fig 2A**). Using David bioinformatic resources [24, 25], we also observed an enrichment of a
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58
59 270 subset of proteins associated to SCRIB that are involved in Wnt/ β -catenin signaling, in
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3 271 particular members of the β -catenin destruction complex which includes APC, Axin,
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5 272 AMER1, DVL and GSK3 β (**Fig. 2B**). Further analysis using STRING ‘functional protein
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7 273 association networks’ [26] highlighted a protein-protein association network associated to
8
9 274 SCRIB (**Fig. 2C**). We thus evidence a highly connected network of 17 proteins associated to
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11 275 SCRIB which comprise 10 proteins involved in Wnt/ β -catenin signaling (**Fig. 2B**).
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17 277 **Wnt3a stimulation promotes SCRIB association with β -catenin**

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19 278 To confirm the mass spectrometry data, we conducted immunoprecipitation of
20
21 279 endogenous SCRIB using specific antibody, and monitored the presence of
22
23 280 endogenous β -catenin by western blot analysis. We observed that SCRIB and β -catenin
24
25 281 association was promoted under MG132 treatment at two different time points and at two
26
27 282 different concentrations (**Fig 3A**). We also observed a modification of the migration pattern of
28
29 283 β -catenin in polyacrylamide gels which is typical of ubiquitinated forms, suggesting that
30
31 284 SCRIB might promote degradation of β -catenin through its association with the destruction
32
33 285 complex (**Fig. 3A**). As the destruction complex is inhibited by Wnt stimulation, we next
34
35 286 stimulated cells overnight with Wnt3a conditioned media and conducted immunoprecipitation
36
37 287 of endogenous SCRIB and looked at the presence of β -catenin in the SCRIB complex. We
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39 288 observed that Wnt3a stimulation promotes the association between SCRIB and β -catenin by
40
41 289 1.8 fold (**Fig. 3B**). To further confirm this observation, we treated cells with LiCl, an inhibitor
42
43 290 of GSK3 β leading to β -catenin stabilization, and assessed endogenous SCRIB/ β -catenin co-
44
45 291 immunoprecipitation. We observed that the association between SCRIB and β -catenin is
46
47 292 potentiated when GSK3 β is inhibited, suggesting that this interaction is regulated by
48
49 293 activation of Wnt/ β -catenin signaling (**Fig. 3C**). We further assessed the colocalization
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51 294 between SCRIB and β -catenin in HEK 293T cells by immunofluorescence. We observed that
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3 295 both proteins colocalize at the plasma membrane with no difference in their subcellular
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5 296 localization following Wnt3a conditioned media or LiCl treatments (**Fig. 3D**). Taken together,
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8 297 our data suggest that SCRIB is associated with β -catenin under basal conditions. However,
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10 298 this interaction is promoted when β -catenin is stabilized by Wnt3a or LiCl treatments.

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14 15 300 **SCRIB is a negative regulator of the Wnt/ β -catenin signaling**

16
17 301 Since SCRIB is associated with β -catenin and this interaction is modulated by Wnt3a
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19
20 302 stimulation, we next asked whether SCRIB can act as a modulator of Wnt/ β -catenin signaling.
21
22 303 We first screened siRNAs able to downregulate SCRIB in human cells. We identified two
23
24 304 independent siRNAs able to decrease SCRIB expression by at least 50% (**Fig. 4A**). HEK293T
25
26 305 and MCF10A cell lines stably expressing a β -catenin luciferase reporter and
27
28
29 306 a *Renilla* luciferase control protein were then transfected with either control or SCRIB
30
31 307 siRNAs. In the control transfected MCF10A and HEK293T cells, addition of Wnt3a led to 7-
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33 308 and 71-fold activity of the reporter, respectively, (**Fig. 4B and Fig. 4C**) compared to cells
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35
36 309 treated with control conditioned medium. Depletion of SCRIB led to an increase in Wnt3a-
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38 310 mediated activation by 21- and 170-fold in MCF10A and HEK293T cells, respectively (**Fig.**
39
40 311 **4B and Fig. 4C**). We therefore conclude that SCRIB acts as a negative regulator of Wnt
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42
43 312 signaling. It has been previously shown that SCRIB associates to β -catenin through its PDZ
44
45 313 domains [36]. To further investigate the role of SCRIB as a negative regulator of Wnt/ β -
46
47
48 314 catenin signaling, we overexpressed full length SCRIB fused to GFP in HEK293T cells and
49
50 315 observed a partial decrease of activity of the luciferase reporter (**Fig. 4D**). However, when we
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53 316 overexpressed a mutant form of SCRIB lacking its PDZ domains (GFP-SCRIB Δ PDZ), we did
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55 317 not observe any difference with control conditions, confirming that the PDZ domains of
56
57 318 SCRIB are required for its repressive function (**Fig. 4D**). Expression levels of GFP-SCRIB
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59 319 and GFP-SCRIB Δ PDZ were controlled by western blot analysis (**Fig. 4E**). Together our data

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3 320 demonstrate the importance of SCRIB as a negative regulator of Wnt/ β -catenin signaling,
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6 321 probably through its direct interaction with β -catenin.
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9
10 323 **Discussion**

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12 324 We and others have demonstrated the importance of SCRIB and LANO in cancer
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15 325 development [4, 9, 13, 37]. In this study, we identified large protein complexes associated
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17 326 with these two paralogs and carried out for the first-time a direct comparison of their
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19 327 associated protein complexes. Using an established functional proteomic approach, our results
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21 328 show that SCRIB and LANO interact with a common set of 199 proteins. Furthermore, we
22
23 329 demonstrate that LANO, which has high sequence homology with SCRIB in its N-terminal
24
25 330 region (**Fig. 1A**), interacts with a specific set of 275 proteins which include 4 PDZ domain-
26
27 331 containing proteins (DLG1, CASK, MPP7, and SNX27) and SCRIB with 191 specific partners
28
29 332 which include 6 PDZ containing proteins (PDZ8, GOPC, SIPA1L1, SIPA1L2, SIPA1L3,
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31 333 PTPN13). We further characterized proteins associated with SCRIB in conditions where we
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33 334 inhibited proteasome activity and found that this treatment led to the association between
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35 335 SCRIB and the β -catenin destruction complex. Further analysis revealed that SCRIB is a
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37 336 negative regulator of the Wnt/ β -catenin signaling pathway. Our data suggest that SCRIB
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39 337 scaffolds components of the destruction complex and most probably inhibits the membrane
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41 338 pool of β -catenin (**Fig. 3D**). Indeed, downregulation of SCRIB expression promotes an
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43 339 increase of Wnt/ β -catenin signaling using reporter assays. In conclusion, our data suggest that
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45 340 SCRIB may control Wnt signaling in epithelial cells.

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47 341 Our study deciphers, for the first time, the protein complexes associated with proteins
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49 342 encoded by the two close paralogues *SCRIB* and *LANO*. We demonstrate that SCRIB and
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51 343 LANO can associate since specific LANO peptides have been identified by LC-MS in the
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53 344 SCRIB complex (**Fig. 1B**) and conversely that specific SCRIB peptides were found in the
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3 345 LANO complex (**Fig. 1C**). The SCRIB-LANO interaction was further confirmed by
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5 346 coimmunoprecipitation (**Fig. 1D**). Interestingly, we have recently demonstrated that LANO
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7 347 and SCRIB colocalize at the basolateral membranes of epithelial cells [38]. LANO harbors a
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9 348 C-terminal PDZ binding motif which allows its interaction with a subset of PDZ domain-
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11 349 containing proteins including DLG1, SNX27 or SCRIB (**Fig. 1A**) [1]. Although contribution
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13 350 of LANO to SCRIB functions or vice-versa has not yet been studied, we show here and in a
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15 351 recent report [38] that both proteins negatively regulate Wnt/ β -catenin signaling suggesting
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17 352 overlapping functions. Our extensive analysis of their associated protein complexes will help
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19 353 in the future at a better understanding of their respective role in epithelial cells. Interestingly,
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21 354 *Scrib* knock out mice display morphogenetic defects [39] whereas *Lano* deficient mice are
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23 355 viable and fertile with no obvious phenotype [38]. These genetic results suggest that the two
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25 356 paralogues contribute differently to murine embryonic development and, combined to the
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27 357 results of our comparative interactomes, that SCRIB and LANO have probably both
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29 358 redundant and non-redundant functions. It would be of great interest in the future to cross
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31 359 *scrib* and *lano* deficient mice and assess a possible functional interaction between these genes.
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33 360 A functional proteomic approach has been used to demonstrate that SCRIB is associated with
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35 361 a specific protein complex involved in Wnt/ β -catenin signaling pathway. Our results suggest
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37 362 that SCRIB is a negative modulator of Wnt/ β -catenin signaling. It has been recently shown
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39 363 that SCRIB, when localized at the plasma membrane, recruits β -TRCP, an E3-ubiquitin ligase
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41 364 that promotes degradation of TAZ in a proteasome-dependent manner, leading to inhibition of
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43 365 the HIPPO signaling pathway [7]. Furthermore, TAZ is also a component of the β -catenin
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45 366 destruction complex which, through recruitment of β -TRCP, degrades β -catenin [40]. Our
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47 367 initial proteomic approach done under basal conditions (**Fig. 1B**) failed to identify
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49 368 components of HIPPO or Wnt signaling pathways associated to SCRIB. However, by
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51 369 coimmunoprecipitation and western blot analysis of endogenous SCRIB, we could recover a
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3 370 SCRIB/ β -catenin (**Fig. 3**) but not SCRIB/TAZ complex (data not shown) suggesting that very
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6 371 few β -catenin is associated to SCRIB under basal conditions. Under MG132 treatment, we
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8 372 were able to easily identify by mass spectrometry β -catenin and members of the β -catenin
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10 373 (but still not TAZ) destruction complex associated with SCRIB demonstrating that, at least in
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12 374 our cellular model systems, the SCRIB/ β -catenin destruction complex is predominant and
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14 375 regulated directly or indirectly by proteosomal degradation. We next showed that SCRIB
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16 376 behaves as a negative modulator of this signaling pathway as it does with AKT, ERK or
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18 377 HIPPO pathways (**Fig. 4B**). It has been recently shown that LANO represses Wnt/ β -catenin
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20 378 signaling and promotes the maintenance of breast cancer stem cells [38]. It would be
21
22 379 interesting to assess a possible functional cooperation between the two paralogues in this
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24 380 setting (**Fig 1D**).

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29 381 Finally, β -catenin is a central component of the cadherin cell adhesion complex and plays an
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31 382 essential role in the maintenance of the epithelial structures [41]. Studies have shown that
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33 383 SCRIB subcellular localization is altered in colorectal carcinoma and correlates with
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35 384 intracellular accumulation of β -catenin compared to normal adjacent epithelia [42].
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37 385 Furthermore, it has been recently shown that SCRIB is required for β -catenin localization at
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39 386 the plasma membrane in order to maintain the integrity of tight junctions [36]. Our findings
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41 387 suggest that deregulation of expression of SCRIB may potentiate Wnt/ β -catenin signaling and
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43 388 promotes cancer development.

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3 390 **Figure legends**
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5 391 **Figure 1 Protein landscapes associated to SCRIB and LANO**
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7 392 **(A)** Topology of SCRIB and LANO showing sequence homologies. **(B-C)** Mass spectrometry
8 393 analysis of the protein complexes after FLAG immunopurification of proteins from HEK293T
9 394 cells expressing FLAG-SCRIB **(B)** or FLAG-LANO **(C)**. Volcano plots showing differential
10 395 Log₂(iBAQ intensity) levels (x axis) and $-\text{Log}(\text{p-value})$ (y axis) for SCRIB versus control **(B)**
11 396 and LANO versus control **(C)**. The full lines are indicative of significant hits obtained at a
12 397 Log₂ difference of 2 and a $-\text{Log p-value}$ of 2 and are highlighted in green for FLAG-SCRIB
13 398 versus control conditions **(B)**, in red for FLAG-LANO versus control conditions **(C)** and in
14 399 grey for proteins with non-significant changes. **(D)** Immunopurification of FLAG-SCRIB
15 400 (lane 3) or FLAG-LANO (lane 4) extracted from HEK293T cell lysates using anti-FLAG
16 401 antibody coupled to sepharose beads allows the identification of a SCRIB (red arrow)-LANO
17 402 (green arrow) interaction. Expression of FLAG-SCRIB and FLAG-LANO in lysates are
18 403 shown lane 1 and lane 2 respectively **(E)** Venn diagram showing number of total proteins (n)
19 404 or PDZ domain proteins (n (PDZ)) associated to SCRIB and/or LANO. **(B-C)** Known SCRIB
20 405 and LANO interactors indexed in BioGRID are highlighted in red and PDZ proteins are
21 406 highlighted in orange.
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44 408 **Figure 2 SCRIB associates to the β -catenin destruction complex**
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46 409 **A)** Volcano plots showing differential Log₂(LFQ intensity) levels (x axis) and $-\text{Log}(\text{p-value})$
47 410 (y axis) for proteins associated to SCRIB under DMSO or MG132 treatments. The dotted
48 411 lines are indicative of significant hits obtained at a Log₂ difference of 2 and a $-\text{Log p-value}$ of
49 412 2 and are highlighted in blue for FLAG-SCRIB under DMSO vs MG132 treatments. Grey
50 413 spots correspond to proteins with non-significant changes. Of note, we did not observe
51 414 variation of SCRIB expression by MS analysis following MG132 treatment. **(B)** Top
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3 415 Biological process enrichment using proteins found regulated under MG132 treatment vs
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5 416 DMSO using a biocomputational tool (DAVID). (C) Scheme of functional protein networks
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7 417 using STRING's website with the SCRIB-associated proteins regulated by MG132 treatment
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9 418 and including SCRIB as bait. One main cluster (red) with highly connected proteins (green
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11 and blue) is shown.
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17 421 **Figure 3 Wnt3a stimulation promotes SCRIB association with β -catenin**

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19 422 (A-C) Immunopurification of endogenous SCRIB and presence of β -catenin in the complex
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21 423 was assessed by western blot analysis. Before lysis, cells were treated with MG132 for the
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23 424 indicated times and concentrations (A). Cells were treated with Ctl- or Wnt3a-condition
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25 425 media for 16 hours before lysis (B). Cells were treated with 10mM LiCl or 10mM NaCl for
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27 426 16 hours before lysis (C). (D) HEK 293T cells were seeded on Poly-L-Lysine coverslips and
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29 427 treated with 10mM NaCl, 10mM LiCl, Ctl-CM or Wnt3a-CM for 16 hours. After fixation,
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31 428 cells were stained with phalloidin (purple) to reveal polymerized actin, anti-SCRIB antibody
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33 429 (green), anti- β -catenin antibody (red) and DAPI (blue). Scale bars 20 μ M.
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40 431 **Figure 4 SCRIB is a negative regulator of Wnt/ β -catenin signaling**

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42 432 (A) Two independent siRNAs targeting SCRIB or a control siRNA (Non-Targeting, NT) were
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44 433 transfected into HEK 293T cells. Decrease of SCRIB expression was confirmed by western
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46 434 blot analysis. (B-C) MCF10A (B) or HEK 293T (C) cells stably expressing the TOP Flash
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48 435 luciferase reporter were transfected with the indicated siRNAs and, after 24 hours, were
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50 436 treated with either WT-CM or Wnt3a-CM for 16 hours. The luciferase reporter activity was
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52 437 measured with a luminometer (mean \pm standard deviation; $n = 3$). (D) HEK 293T cells
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54 438 transfected with the indicated constructs were treated with WT-CM or Wnt3a-CM for 16
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56 439 hours. Expression of GFP-SCRIB constructs was assessed by western blot analysis (E).
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3 549 **Conflict of interest statement**
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5 550 The authors have no conflict of interest to declare.
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24 565 assistance in mass spectrometry analysis.
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46 567 **Authors' contributions**
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48 568 AMD designed the study. JPB and LC supervised the study. AMD, JPB and LC wrote the
49 569 manuscript. AMD and MSW performed molecular, cellular and biochemical experiments.
50 570 AW performed immunofluorescence experiments. SA, EB, LC and AMD performed the mass
51 571 spectrometry analysis.
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573 **Supplementary Materials and Methods**

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575 **Mass spectrometry analysis.**

576 Protein extract were loaded on NuPAGE 4-12% Bis-Tris acrylamide gels (Life Technologies)
577 to stack proteins in a single band that was stained with Imperial Blue (Pierce, Rockford, IL)
578 and cut from the gel. Gels pieces were submitted to an in-gel trypsin digestion¹ with slight
579 modifications. Briefly, gel pieces were washed and destained using 100 mM
580 NH_4HCO_3 /acetonitrile (50/50). Destained gel pieces were shrunk with acetonitrile and were
581 re-swollen in the presence of 100 mM ammonium bicarbonate in 50% acetonitrile and dried at
582 room temperature. Protein bands were then rehydrated and cysteines were reduced using 10
583 mM DTT in 100 mM ammonium bicarbonate pH 8.0 for 45 min at 56 C before alkylation in
584 the presence of 55 mM iodoacetamide in 100 mM ammonium bicarbonate pH 8.0 for 30 min
585 at room temperature in the dark. Proteins were then washed twice in 100 mM ammonium
586 bicarbonate and finally shrunk by incubation for 5 min with 100 mM ammonium bicarbonate
587 in 50% acetonitrile. The resulting alkylated gel pieces were dried at room temperature. The
588 dried gel pieces were re-swollen by incubation in 100 mM ammonium bicarbonate pH 8.0
589 supplemented with trypsin (12.5 ng/ μL ; Promega) for 1h at 4 C and then incubated overnight
590 at 37°C. Peptides were harvested by collecting the initial digestion solution and carrying out
591 two extractions; first in 5% formic acid and then in 5% formic acid in 60% acetonitrile.
592 Pooled extracts were dried down in a centrifugal vacuum system. Samples were reconstituted
593 with 0.1% trifluoroacetic acid in 4% acetonitrile and analyzed by liquid chromatography
594 (LC)-tandem mass spectrometry (MS/MS) using a LTQ-Orbitrap Velos Mass Spectrometer
595 (Thermo Electron, Bremen, Germany) or using an Orbitrap Fusion Lumos Tribrid Mass
596 Spectrometer (Thermo Electron, Bremen, Germany) both online with a nanoRSLC Ultimate
597 3000 chromatography system (Dionex, Sunnyvale, CA). Peptides were separated on a Dionex
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3 598 Acclaim PepMap RSLC C18 column. First peptides were concentrated and purified on a pre-
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5 599 column from Dionex (C18 PepMap100, 2 cm × 100 μm I.D, 100 Å pore size, 5 μm particle
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7 600 size) in solvent A (0.1% formic acid in 2% acetonitrile). In the second step, peptides were
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9 601 separated on a reverse phase LC EASY-Spray C18 column from Dionex (PepMap RSLC
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11 602 C18, 15 or 50 cm × 75 μm I.D, 100 Å pore size, 2 μm particle size) at 300 nL/min flow rate.
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13 603 After column equilibration using 4% of solvent B (20% water - 80% acetonitrile - 0.1%
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15 604 formic acid), peptides were eluted from the analytical column by a two steps linear gradient
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17 605 (4-20% acetonitrile/H₂O; 0.1 % formic acid for 90 min and 20-45% acetonitrile/H₂O; 0.1%
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19 606 formic acid for 30 min). For peptide ionization in the EASY-Spray nanosource in front of the
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21 607 LTQ-Orbitrap Velos, spray voltage was set at 1.9 kV and the capillary temperature at 275°C.
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23 608 The Orbitrap Velos was used in data dependent mode to switch consistently between MS and
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25 609 MS/MS. Time between Masters MS spectra were acquired with the Orbitrap in the range of
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27 610 *m/z* 300-1700 at a FWHM resolution of 30 000 measured at 400 *m/z*. For internal mass
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29 611 calibration, the 445.120025 ion was used as lock mass. The more abundant precursor ions
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31 612 were selected and collision-induced dissociation fragmentation was performed in the ion trap
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33 613 on the 10 most intense precursor ions measured to have maximum sensitivity and yield a
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35 614 maximum amount of MS/MS data. The signal threshold for an MS/MS event was set to 5000
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37 615 counts. Charge state screening was enabled to exclude precursors with 0 and 1 charge states.
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39 616 Dynamic exclusion was enabled with a repeat count of 1, exclusion list size 500 and exclusion
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41 617 duration of 30 s.
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45 619 For peptide ionization in the EASY-Spray nanosource in front of the Orbitrap Fusion Lumos
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47 620 Tribrid Mass Spectrometer, spray voltage was set at 2.2 kV and the capillary temperature at
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49 621 275 °C. The Orbitrap Lumos was used in data dependent mode to switch consistently between
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51 622 MS and MS/MS. Time between Masters Scans was set to 3 seconds. MS spectra were
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3 623 acquired with the Orbitrap in the range of m/z 400-1600 at a FWHM resolution of 120 000
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5 624 measured at 400 m/z . AGC target was set at 4.0×10^5 with a 50 ms Maximum Injection Time. For
6
7 625 internal mass calibration the 445.120025 ions were used as lock mass. The more abundant
8
9
10 626 precursor ions were selected and collision-induced dissociation fragmentation was performed
11
12 627 in the ion trap to have maximum sensitivity and yield a maximum amount of MS/MS data.
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14 628 Number of precursor ions was automatically defined along run in 3s windows using the
15
16 629 “Inject Ions for All Available parallelizable time option” with a maximum injection time of
17
18 630 300 ms. The signal threshold for an MS/MS event was set to 5000 counts. Charge state
19
20 631 screening was enabled to exclude precursors with 0 and 1 charge states. Dynamic exclusion
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22 632 was enabled with a repeat count of 1 and a duration of 60 s.
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27 28 634 **Protein identification and quantification.**

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30 635 Relative intensity-based label-free quantification (LFQ) and iBAQ intensity were processed
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32 636 using the MaxLFQ algorithm[20] from the freely available MaxQuant computational
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34 637 proteomics platform, version 1.6.2.1.[21] The acquired raw LC Orbitrap MS data were first
35
36 638 processed using the integrated Andromeda search engine.[22] Spectra were searched against
37
38 639 the human database subset of the Swiss-Prot database (date 18/09/19, 20394 entries). This
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40 640 database was supplemented with a set of 245 frequently observed contaminants. The
41
42 641 following parameters were used for searches: (i) trypsin allowing cleavage before proline; (ii)
43
44 642 two missed cleavages were allowed; (ii) monoisotopic precursor tolerance of 20 ppm in the
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46 643 first search used for recalibration, followed by 4.5 ppm for the main search and 0.5 Da for
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48 644 fragment ions from MS/MS ; (iii) cysteine carbamidomethylation (+57.02146) as a fixed
49
50 645 modification and methionine oxidation (+15.99491) and N-terminal acetylation (+42.0106) as
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52 646 variable modifications; (iv) a maximum of five modifications per peptide allowed; and (v)
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54 647 minimum peptide length was 7 amino acids and a maximum mass of 4,600 Da. The match
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3 648 between runs option was enabled to transfer identifications across different LC-MS/MS
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5 649 replicates based on their masses and retention time within a match time window of 0.7 min
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8 650 and using an alignment time window of 20 min. The quantification was performed using a
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10 651 minimum ratio count of 1 (unique+razor) and the second peptide option to allow
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12 652 identification of two co-fragmented co-eluting peptides with similar masses. The false
13
14 653 discovery rate (FDR) at the peptide and protein levels were set to 1% and determined by
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16 654 searching a reverse database. For protein grouping, all proteins that cannot be distinguished
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18 655 based on their identified peptides were assembled into a single entry according to the
19
20
21 656 MaxQuant rules. The statistical analysis was done with Perseus program (version 1.6.1.3)
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23 657 from the MaxQuant environment (www.maxquant.org). The LFQ normalised intensities or
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25 658 the iBAQ intensity[30] were uploaded from the ProteinGroups.txt file. The iBAQ intensities
26
27 659 are roughly proportional to the molar quantities of the proteins. Proteins marked as
28
29 660 contaminant, reverse hits, and “only identified by site” were discarded. Quantifiable proteins
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31 661 were defined as those detected in at least 100% of samples in at least one condition. Protein
32
33 662 LFQ normalized intensities were base 2 logarithmized to obtain a normal distribution.
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35 663 Missing values were replaced using data imputation by randomly selecting from a normal
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37 664 distribution centred on the lower edge of the intensity values that simulates signals of low
38
39 665 abundant proteins using default parameters (a downshift of 1.8 standard deviation and a width
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41 666 of 0.3 of the original distribution). In this way, imputation of missing values in the controls
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43 667 allows statistical comparison of protein abundances that are present only in the inhibitors
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45 668 samples. To determine whether a given detected protein was specifically differential a two-
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47 669 sample t-test were done using permutation based FDR-controlled at 0.01 and employing 250
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49 670 permutations. The differential proteomics analysis was carried out on identified proteins after
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51 671 removal of proteins only identified with modified peptides, peptides shared with other
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53 672 proteins, proteins from contaminant database and proteins which are present in 100% in at
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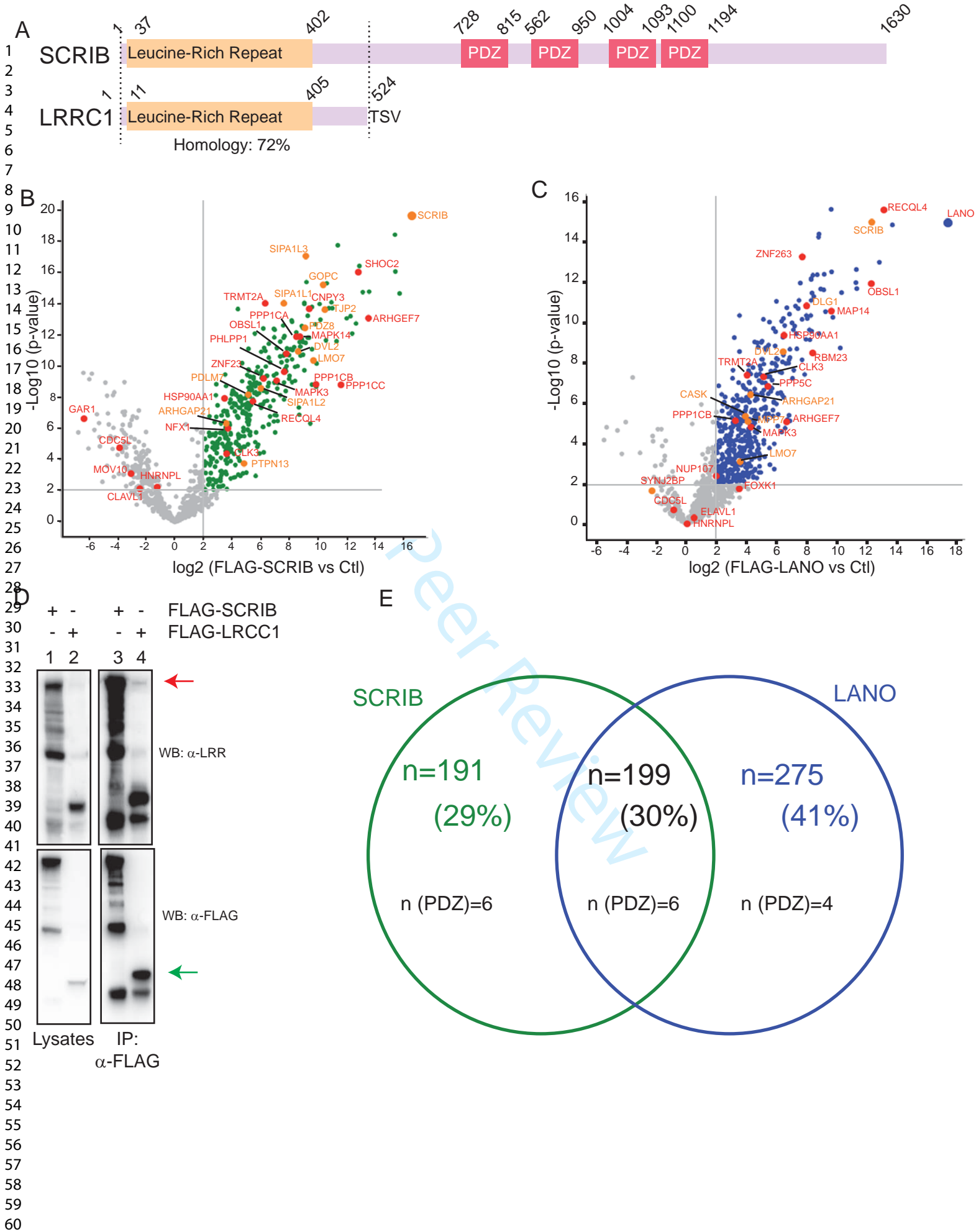
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3 673 least one condition. Alternatively, The iBAQ (Intensity Based Absolute Quantification)[23]
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5 674 were uploaded from the proteinGroups.txt file and transformed by a base logarithm 2 to
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7 675 obtain a normal distribution. Thus, iBAQ intensities are roughly proportional to the molar
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10 676 quantities of the proteins.
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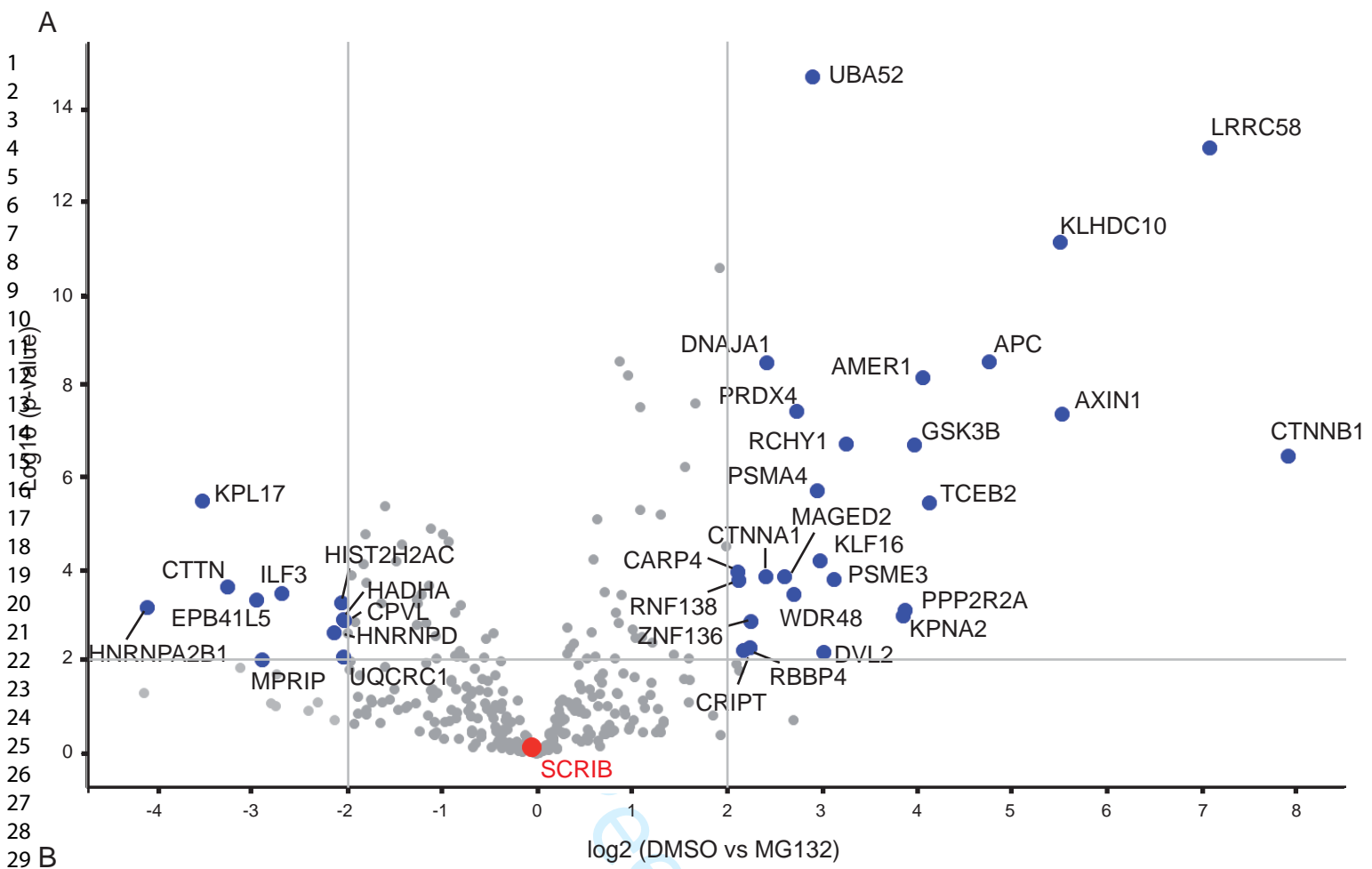
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For Peer Review





B

Biological Process term	AMER1	APC	AXIN1	CTNNB1	DVL2	GSK3B	PSME3	PSME4	RNF138	UBA52	Count	P-value
negative regulation of canonical Wnt signaling pathway	■	■	■	■	■	■	■	■	■	■	8	0.0001
proteasome-mediated ubiquitin-dependent protein catabolic process	■	■	■	■	■	■	■	■	■	■	8	0.00019
beta-catenin destruction complex disassembly	■	■	■	■	■	■	■	■	■	■	6	0.0002
Wnt signaling pathway	■	■	■	■	■	■	■	■	■	■	8	0.00084
positive regulation of canonical Wnt signaling pathway	■	■	■	■	■	■	■	■	■	■	6	0.001
canonical Wnt signaling pathway	■	■	■	■	■	■	■	■	■	■	5	0.0015
beta-catenin destruction complex assembly	■	■	■	■	■	■	■	■	■	■	4	0.0043
positive regulation of protein catabolic process	■	■	■	■	■	■	■	■	■	■	3	0.032
Wnt signaling pathway, planar cell polarity pathway	■	■	■	■	■	■	■	■	■	■	4	0.045

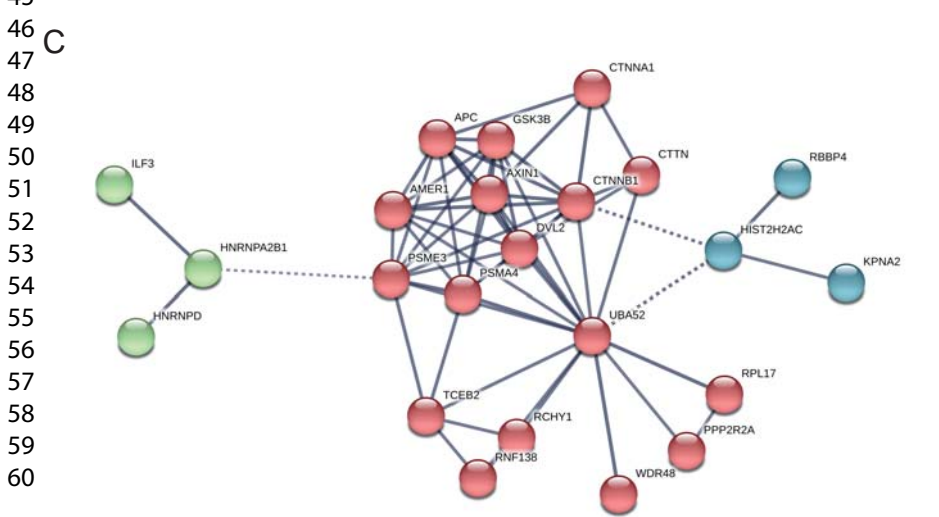


FIGURE 3

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