Supporting Information

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SI Text

Sequence Analysis and Modeling. The model of the DCNL3 PONY domain was generated based on the structure of scDcn1 (1, 2) through the Swiss Model Server and modified by using the software COOT. Cul sequences were aligned by using the Vector NTI program (Invitrogen). For the multiple sequence alignment of DCNL3 N-terminal domain, the MAFFT program was used (3). Myristoylation of DCNL3 was predicted with "medium confidence" and "reliable," respectively, by analyzing the DCNL3 amino acid sequence (GenBank accession no. NP_775746) by using the algorithms at www.expasy.org/tools/myristoylator (4) and http://mendel.imp.ac.at/myristate/SUPLpredictor.htm (5).

Yeast Experiments. Yeast expression was achieved by cloning DCNL-HA cDNAs or *DCN1* into p413GPD or p423ADH (6), respectively, followed by transformation into *dcn1*Δ or *rub1*Δ strains (S288C background). Exponentially growing yeast cultures were lyzed by using the Fastprep system (Qbiogen), and the neddylation state of Cdc53 was examined by immunoblotting with polyclonal Cdc53 antibodies (Santa Cruz).

cDNAs and Primers. To clone the human Dcn1-like proteins, the following IMAGE clones were used as cDNA templates (IMAGE clone cDNA no. is in parentheses): DCNL1 (3939758), DCNL2–1 and DCNL2–2 (6494926), DCNL3 (5590225), and DCNL5 (2821548).

Details of primers used for cloning and RT-PCR are available on request.

RT-PCR. mRNA levels were compared by quantitative RT-PCR. To extract mRNA from cells, cells were lyzed with QIAshredder columns (Qiagen), RNA was isolated with RNeasy kit (Qiagen), and mRNA was reverse-transcribed to cDNA with RNase H-Reverse transcriptase (Invitrogen) and random primers from Microsynth. cDNA levels were quantified by using SYBR Green PCR Master mix and ABI Prism 7000 Sequence Detection System from Applied Biosystems. Individual samples were normalized to the human housekeeping gene GAPDH.

RNAi Oligos. For control RNAi, scrambled siRNA from Microsynth was applied (negative control, AGGUAGUGUAAUCGCCUUGTT). The following siRNA duplexes were used for specific down-regulation of genes: siRNA hUbc12 target region cDNA, GGGCTTCTACAAGAGTGGGAAGT; siRNA DCNL1 target region cDNA, TCCTGAACTTTATATACGA; siRNA DCNL2 target region cDNA, GCGAGAGAACTGCTATCTA; stealth siRNA DCNL3 target region cDNA, CCACAGAATTTCGAGTGCTGCTC.

Antibodies. Antibodies against full-length GST-DCNL2 or GST-DCNL3 were raised in rabbits (Eurogentec) and affinity-purified on MBP-DCNL2 or MBP-DCNL3, respectively. DCNL2 antibodies cross-react with the closely related DCNL1.

The following antibodies were used in this study: Cdc53 (yC-18; Santa Cruz), Cul1 (51–1800; Zymed), Cul2 (71–8700; Zymed), Cul3 (7), Cul4 C-terminal antibody (recognizes Cul4A and Cul4B) (8), Cul5 (H-300; Santa Cruz), Cul7 (Bethyl Laboratories), hUbc12 (A-655; Boston Biochem), Nrf2 (sc-13032; Santa Cruz), CAND1 (sc-10672; Santa Cruz), Flag M2 (F3165; Sigma), HA11 (MMS-101R; Covance), Rbx1 (AVARP03042; AVIVA Systems Biology), α-tubulin (T5169; Sigma), GST (Santa Cruz), His (Qiagen), Na^+/K^+ ATPase (Ab7671; Abcam), HNRPC (hnRNP-C1/C2, R5028; Sigma), Calnexin (a gift from A. Helenius), Caveolin1 (sc894; Santa Cruz), EGFR (MS-400-P1; ThermoScientific), Avidin-HRP (Bio-Rad), and neutravidin-HRP (Pierce).

Protein Purification and in Vitro Binding. GST-tagged human Cul3 complexed with 6×His-tagged Rbx1 was expressed in Sf9 insect cells and purified by using Ni-NTA agarose resin (Qiagen) (7). 6×His-tagged human DCNL1 and DCNL3 wild-type and mutant proteins were expressed and purified from *Escherichia coli* BL21 (DE3) cells. A 20-μL sample containing 200 pmol of GST-Cul3/6×His-Rbx1 complex was mixed with various amounts of wild-type 6×His-DCNL1/3 or mutant proteins, incubated for 1 h at 4 °C, and then captured by 10 μL of glutathione resin in 100 μL of assay buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM DTT]. For human DCNL3 and hUbc12 interaction, GST-tagged Cdc34, UbcH7, and hUbc12 were expressed in *E. coli* BL21 (DE3) cells and immobilized on glutathione resin. Five microliters of glutathione resin containing 200 pmol of GST-hUbc12, UbcH7, or Cdc34 was mixed with 600 pmol of 6×His-DCNL3 and incubated for 1 h at 4 °C in assay buffer. After incubation, the glutathione resin was washed 3 times with 1 mL of assay buffer and then eluted with SDS buffer. Bound proteins were detected by immunoblotting using anti-GST (Santa Cruz) and anti-His antibodies (Qiagen).

Immunoprecipitations. HeLa cells were lyzed by needle extraction in Nonidet P-40 lysis buffer for 30 min at 4 °C. Cell lysates were centrifuged for 10 min at maximum speed in a tabletop centrifuge to remove cell debris. The low-speed extract was incubated for 2 h at 4 °C with precross-linked beads (anti-HA-agarose, A2095; Sigma) or Affiprep protein A beads (BioRad), cross-linked to the antibody of interest by using dimethylpimelimidate dihydrochloride (Sigma), then washed 3 times with TBS-T (0.05% Tween20), 4 times with IP buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 5% glycerol, 20 mM β-glycerophosphate, 1 mM DTT, complete protease inhibitor], and 3 times with TBS-T. Finally, bound proteins were eluted from beads with 100 mM glycine (pH 2) and denatured with 4× SDS loading buffer for 10 min at 95 °C.

Cell Fractionation and Isolation of DRMs. Two 14-cm dishes of HeLa cells or HeLa cells transfected with HA-tagged wild-type or DCNL3 mutants were harvested in sucrose lysis buffer [250 mM sucrose, 20 mM Tris (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM NaF, 20 mM β-glycerophosphate, 1 mM DTT, complete protease inhibitor mixture] and subjected to osmotic pressure for 10 min on ice. After swelling, cells were broken by 10 passes through a 22G needle, and an aliquot was saved for the whole-cell control. The cell lysates were centrifuged twice at 16,000 × *g* for 10 min to obtain a postnuclear lysate. The postnuclear supernatant was centrifuged at 100,000 × *g* for 45 min to separate membrane and cytosol fractions. To enhance the purity of the samples, the remaining supernatant was recentrifuged twice to remove pellet leftovers, while the original pellet after ultracentrifugation was twice resuspended in sucrose lysis buffer [supplemented with DNase (2.7 units/mL) and RNase (40 μg/mL)] followed by ultracentrifugation. The purified cytoplasmic fraction was denatured with 4× SDS sample buffer for 10 min at 95 °C. The purified membrane fraction was resuspended in equal

volume of 1% Triton X-100 buffer [1% Triton X-100, 5% glycerol, 20 mM Tris (pH 7.5), 2 mM EDTA, 1 mM NaF, 20 mM β-glycerophosphate, 1 mM DTT, complete protease inhibitor mixture] for 30 min at 4 °C. The membrane fraction was then subjected to ultracentrifugation and the supernatant containing solubilized membranes was saved and denatured. Equal volumes of purified cytosol and membrane fractions were loaded on a 6–12% SDS gel, while only one-tenth of the whole-cell sample was analyzed.

DRMs were separated from soluble membranes as described (9). Briefly, HeLa cells grown on two 14-cm dishes were lyzed at 4 °C in Triton X-100 buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 50 mM Tris·HCl, pH 8) by homogenization with 25 strokes through a 25G needle. After 30-min incubation on ice (whole-cell sample), the lysate was centrifuged for 5 min at 1,100 × *g* to obtain a postnuclear supernatant. The supernatant was adjusted to a final concentration of 40% sucrose, laid under a 5–35% linear sucrose gradient, and centrifuged in a SW41 rotor for 18 h at 200,000 × *g*. One-milliliter samples were taken from the top and analyzed by immunoblotting for the presence of floating DRMs (marker caveolin-1), while soluble membranes were pelleted to the bottom of the gradient (marker calnexin).

Acylbiotin Exchange Chemistry. The acylbiotin exchange chemistry was performed as described (10). Briefly, HeLa cells transfected with HA-tagged wild-type DCNL3 or DCNL3-C4A-C8A were harvested and lyzed in Triton X-100 buffer containing 10 mM NEM to block free cysteines [0.2% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.5), 5 mM EDTA], followed by Biotin-BMCC (Thermo Scientific) with or without hydroxylamine and finally incubation in only Biotin-BMCC. Hydroxylamine was used to cleave palmitoylation off the modified Cys, which was now able to couple to Biotin-BMCC. To exchange buffers between the different steps, proteins were precipitated with acetone and washed with 70% ethanol before resuspension in SDS buffer [4% SDS, 50 mM Tris (pH 7.5), 5 mM EDTA]. After biotinylation of the samples, DCNL3-HA was immunoprecipitated with HA beads, and eluates were blotted with Avidin-HRP to detect biotinylated DCNL3-HA.

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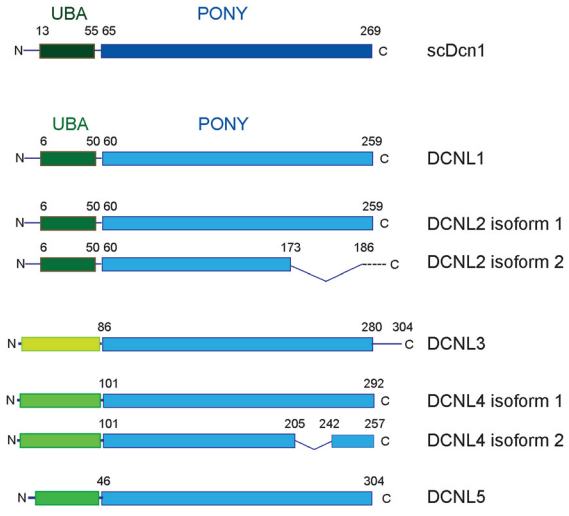
A



B

gene & protein	synonyme protein	synonyme gene	cds accession nbr
DCUN1D1 DCNL1	RP42, SCCRO	RP42, Tes3, SCCRO, DCUN1L1	NM_020640
DCUN1D2 isoform1 DCNL2-1		DCUN1L2, FLJ10704, FLJ20092, C13orf17	NM_001014283
DCUN1D2 isoform 2 DCNL2-2	Acc nbr: Q6PH85	FLJ10704, FLJ20092	
DCUN1D3 DCNL3		44M2.4, FLJ41725, MGC48972	NM_173475
DCUN1D4 isoform 1 DCNL4-1		FLJ42355, KIAA0276	NM_00140402
DCUN1D4 isoform 2 DCNL4-2		FLJ42355, KIAA0276	NM_015115
DCUN1D5 DCNL5		MGC2714, FLJ32431	NM_032299

Fig. S1. Identity of DCNL proteins. (*A*) Schematic representation of the 5 human Dcn1 homologues. The conserved PONY domain is truncated in DCNL2 isoform 2 and DCNL4 isoform 2. Like scDcn1, DCNL1 and DCNL2 possess a UBA domain, while DCNL3, DCNL4, and DCNL5 have distinct amino-terminal extensions. (*B*) A summary of gene, protein names, and accession numbers for human DCNL proteins.

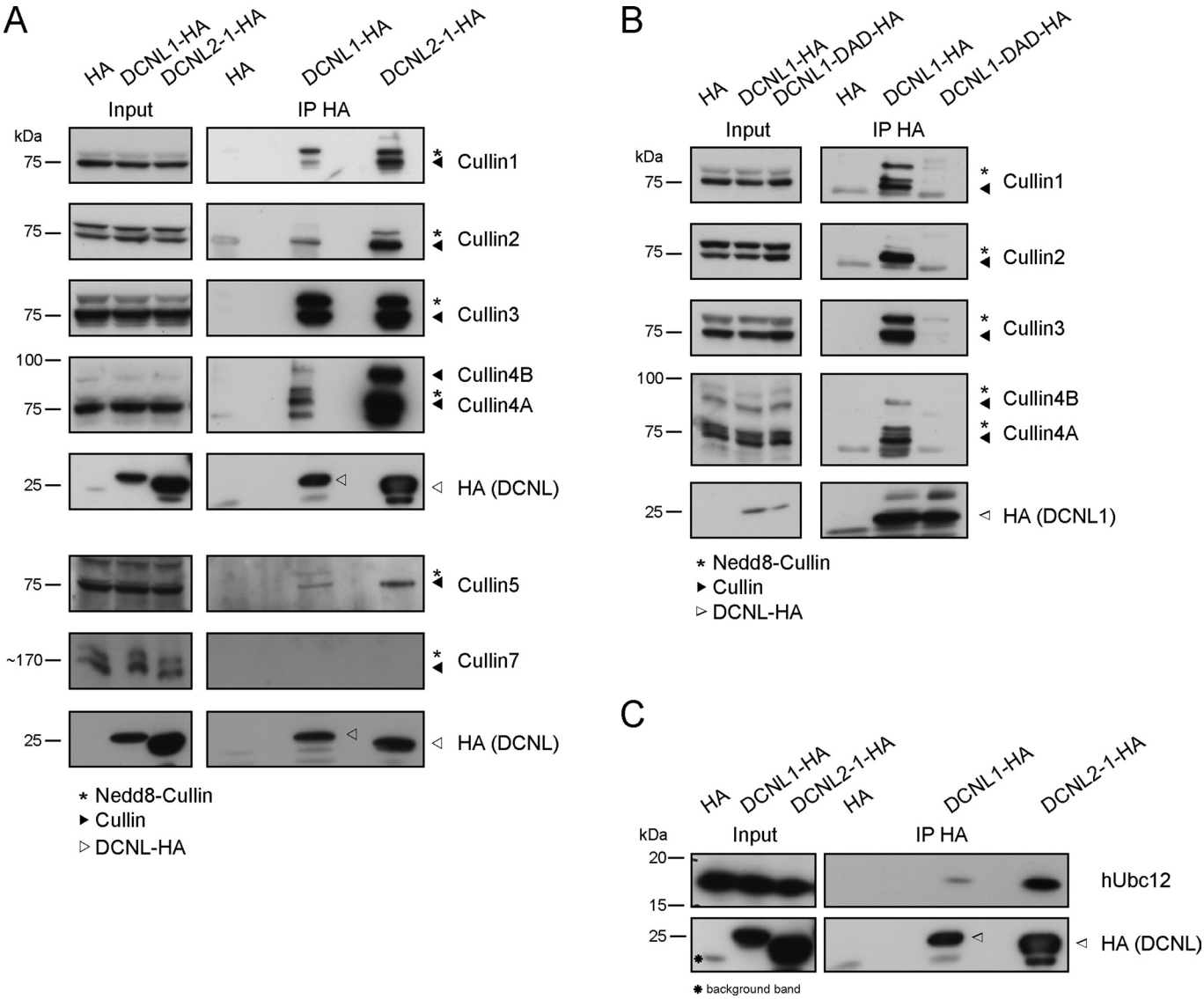
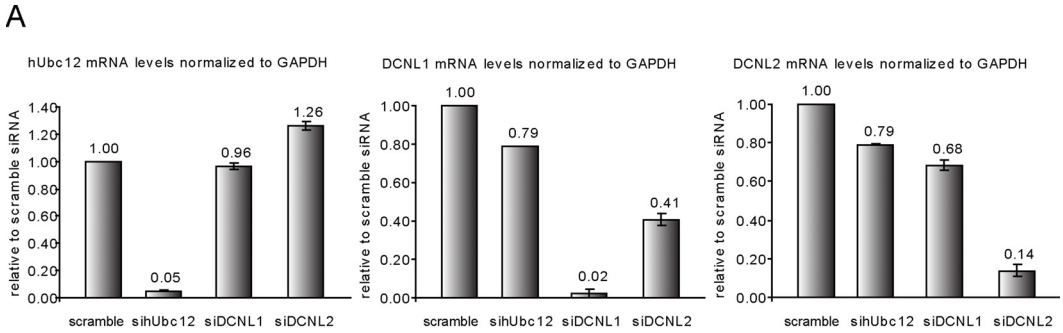
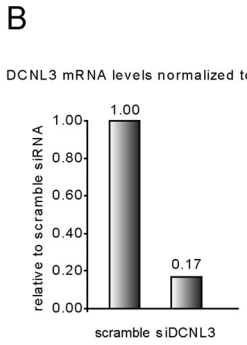


Fig. S2. DCNL1 and DCNL2 bind to human Culs and hUbc12. (*A*) Extracts prepared from HeLa cells expressing either HA-control (HA) or the indicated HA-tagged DCNL were immunoprecipitated with HA-antibodies (IP HA) and associated Culs were analyzed with specific antibodies. An aliquot of the extract before immunoprecipitation is shown at *Left* (input). (*B*) DCNL1-HA or DAD patch mutated DCNL1-DAD-HA stably expressed in 293T-Rex cells were analyzed by immunoprecipitation for their ability to bind endogenous Culs as described in *A*. Note that an intact DAD patch of DCNL1 is required for Cul binding. (C) DCNL1-HA and DCNL2-HA immunoprecipitations were analyzed for interaction with hUbc12 as described in *A*.





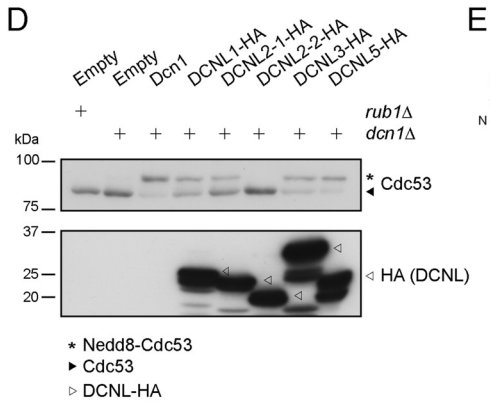
C

Cul1	Mean ratio RNAi/control (neddyl/unneddyl)	Standard deviation	t-test
scramble	1	0	
sihUbc12	0.12	0.04	< 0.001
siDCNL1	0.46	0.10	< 0.001
siDCNL2	0.80	0.20	0.09
siDCNL3	0.40	0.08	< 0.001

Cul3	Mean ratio RNAi/control (neddyl/unneddyl)	Standard deviation	t-test
scramble	1	0	
sihUbc12	0.15	0.02	< 0.001
siDCNL1	0.33	0.08	< 0.001
siDCNL2	0.82	0.12	0.02
siDCNL3	0.56	0.06	< 0.001

Cul2	Mean ratio RNAi/control (neddyl/unneddyl)	Standard deviation	t-test
scramble	1	0	
siDCNL3	0.37	0.15	< 0.001

Cul4A	Mean ratio RNAi/control (neddyl/unneddyl)	Standard deviation	t-test
scramble	1	0	
siDCNL3	0.51	0.11	< 0.001



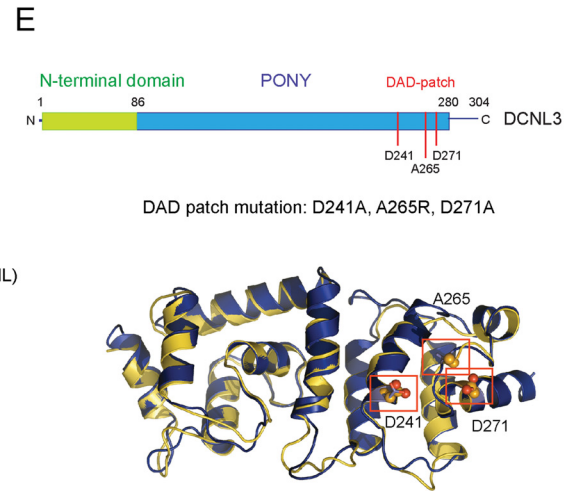


Fig. S3. Yeast complementation assays and effect of DCNL1–3 down-regulation on Cul neddylation in HeLa cells. (*A* and *B*) A fraction of cells used to analyze the neddylation state of endogenous Culs after RNA interference (Fig. 1 *A* and *B*) was used for isolation of total RNA and subsequent quantification of specific mRNA levels by quantitative RT-PCR. Individual panels show the mRNA levels of hUbc12, DCNL1, and DCNL2 (*A*) and DCNL3 (*B*) upon different siRNA treatments. DCNL and hUbc12 mRNA levels were normalized to GAPDH mRNA levels. (*C*) The neddylation state of Cul proteins after RNA interference with DCNL1–3 (Fig. 1 *A* and *B*) was quantified with LI-COR from at least 4 independent experiments. Listed are the mean values and standard deviations of the ratio between specific RNAi and scramble control RNAi of neddylated Cul over unneddylated Cul. Student's *t* test was performed to assess the significance of the difference between scramble control and RNAi for the mean ratio. Values <0.01 are judged as highly significant. (*D*) Yeast *dcn1*Δ or *rub1*Δ cells were transformed with an empty control plasmid (empty), or plasmids expressing as indicated yeast Dcn1 or the indicated human DCNL protein from the constitutive *ADH*- or *GPD*-promoter. Note that the DCNL2–2 splice variant lacks the C-terminal DAD patch (Fig. S1*A*). Cdc53 or the DCNL proteins were detected by immunoblotting of yeast extracts. (*E*) Schematic representation of DCNL3 highlighting the residues comprising the carboxyl-terminal DAD patch. Superimposition of the crystal structure of the scDcn1 PONY domain (blue) with the predicted PONY domain of human DCNL3 (yellow). The positions of the conserved DAD patch residues of scDcn1 (sticks) and human DCNL3 (spheres) are highlighted with red boxes.

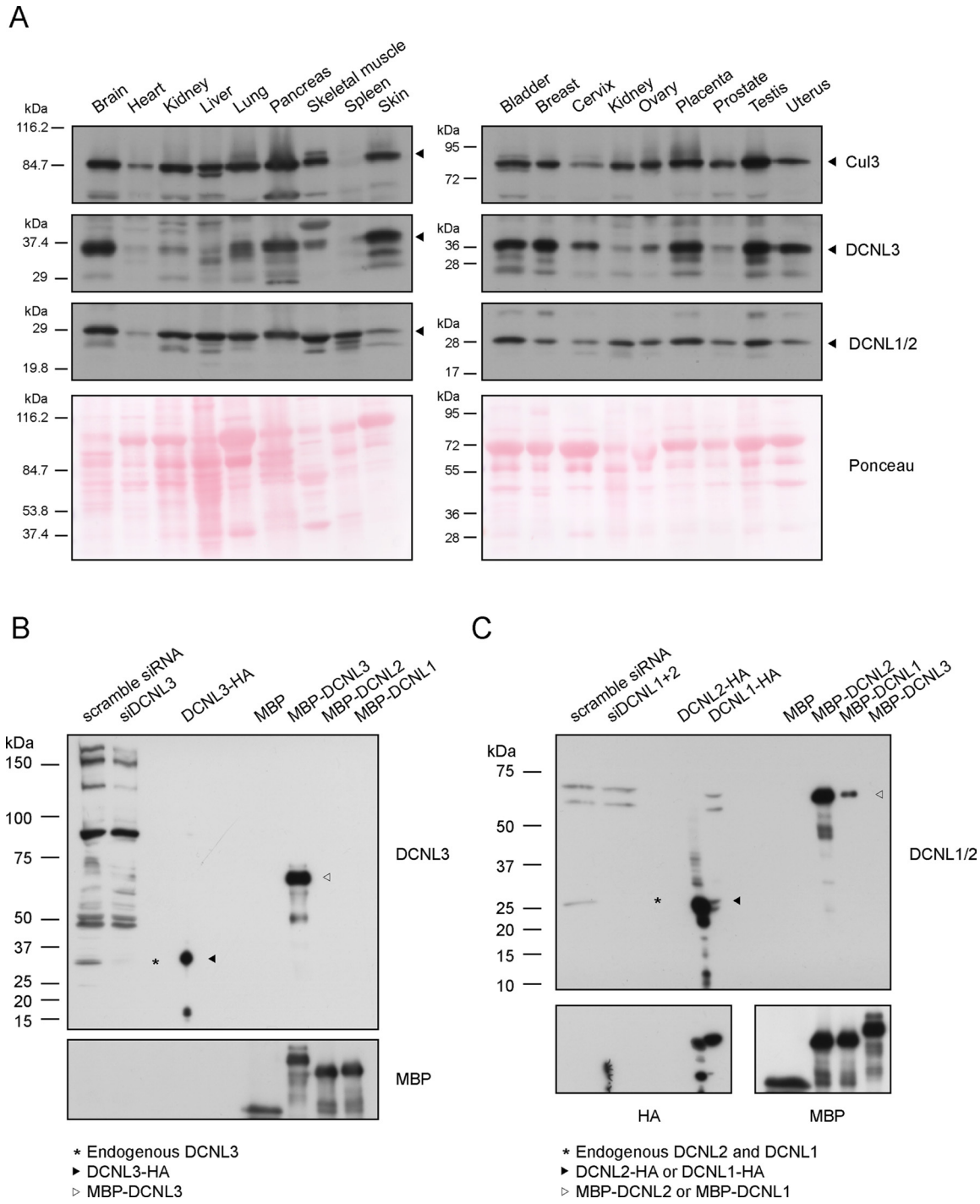


Fig. S4. Characterization of DCNL3 and DCNL1/2 antibodies and the tissue-specific expression of Cul3, DCNL1/2, and DCNL3. (*A*) Human normal tissue blots (ProSci Incorporated) were analyzed for expression of DCNL3, DCNL1/2, and Cul3 by immunoblotting with specific antibodies. Equal loading was controlled by Ponceau S staining. Note that DCNL3 is highly expressed in some ectoderm-derived tissues (e.g., skin) compared with rather low expression in the mesoderm (e.g., spleen or heart), suggesting a developmental role of DCNL3. (*B*) Affinity-purified DCNL3 antibodies were probed on total HeLa extracts, and down-regulated for DCNL3 (lane 2) or scrambled siRNA oligo controls (lane 1). The band identified as DCNL3 (*) migrates slightly faster compared with overexpressed DCNL3-HA (lane 3, filled arrowhead). Ten nanograms of recombinant MBP (lane 4) or MBP-DCNL3 (lane 5, open arrowhead), MBP-DCNL2 (lane 6), and MBP-DCNL1 (lane 7) was probed for recognition with DCNL3 antibodies. Input levels were controlled by immunoblotting with MBP antibodies. (*C*) Affinity-purified DCNL1/2 antibodies cross-react with the closely related DCNL1 and DCNL2. DCNL1/2 antibodies were probed on total HeLa extract, down-regulated for DCNL1 and DCNL2 (lane 2) or scrambled siRNA oligo controls (lane 1). The band down-regulated by RNAi (*) migrates at the same size as DCNL2-HA (lane 3) and DCNL1-HA (line 4), which were both recognized by the antibody (filled arrowhead). Ten nanograms of recombinant MBP (lane 5) or MBP-DCNL2 (lane 6), MBP-DCNL1 (lane 7), MBP-DCNL3 (lane 8) was probed for recognition with DCNL1/2 antibodies (open arrowhead). Input levels were controlled by immunoblotting with HA or MBP antibodies.

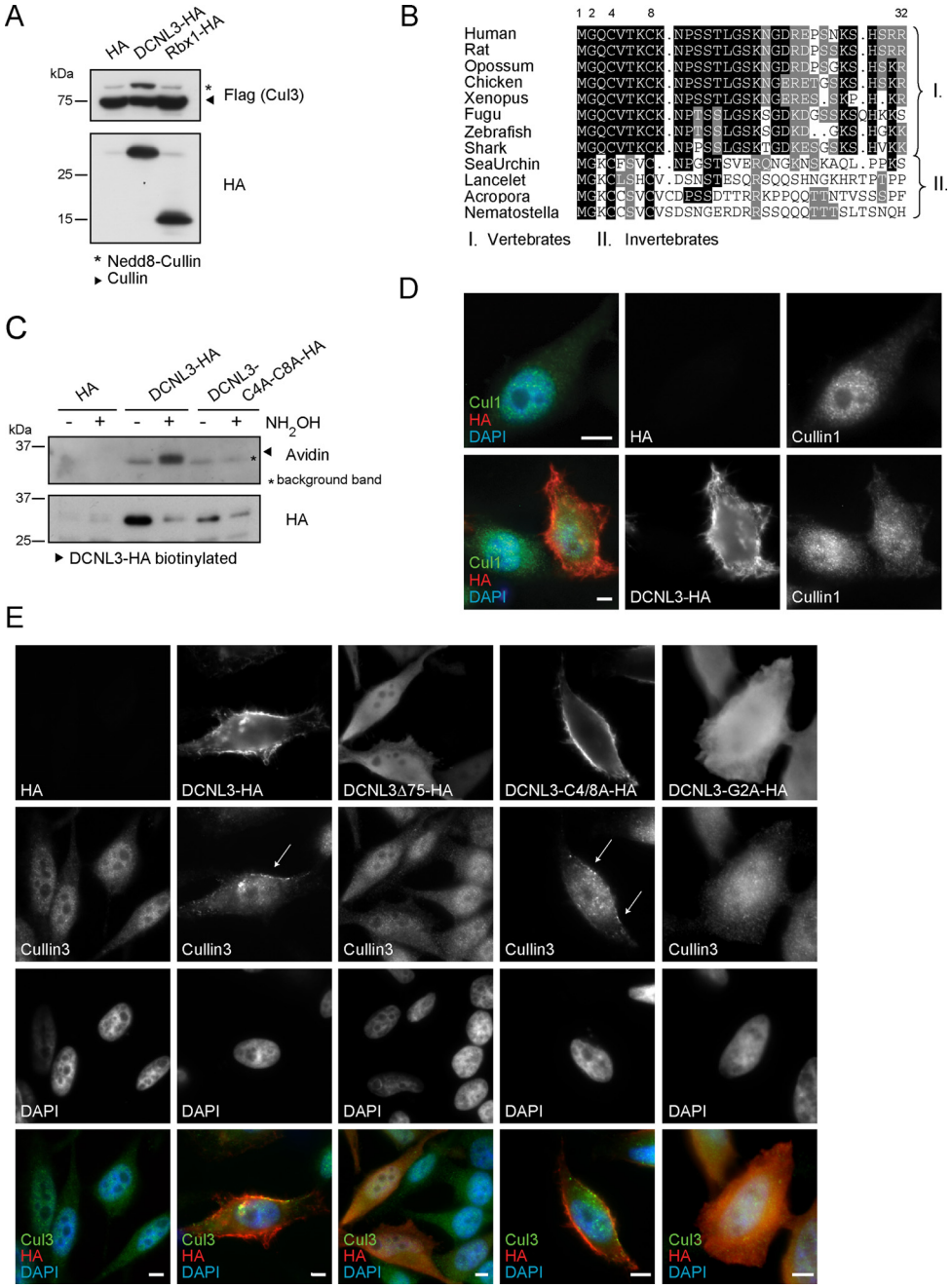
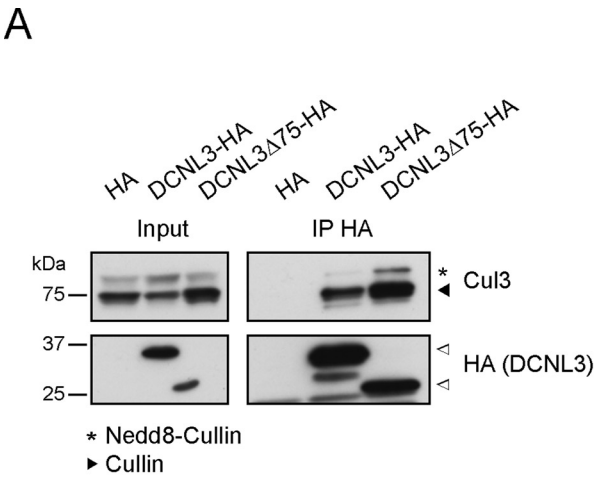
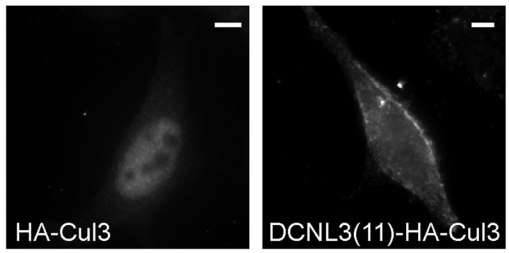


Fig. S5. DCNL3 N-terminal domain is conserved and required for Cul3 recruitment to the membrane. (*A*) HA-tagged DCNL3 or Rbx1 were overexpressed in HeLa cells together with Flag-tagged Cul3. An empty HA plasmid was included as control (HA). The neddylation state was analyzed by immunoblotting (*Upper*), and the expression of DCNL3-HA and Rbx1-HA was controlled by HA antibodies (*Lower*). (*B*) Multiple sequence alignment of human DCNL3 and related DCNL3 from vertebrates and invertebrates shows high conservation of an N-terminal motif (amino acids 1–8). Human (Uniprot entry Q8IWE4; *Homo sapiens*), rat (Uniprot entry Q4V8B2; *Rattus norvegicus*), opossum (Ensembl protein entry ENSMODP00000008768; *Monodelphis domesticus*), chicken (Ensembl protein entry ENSGALP00000003552; *Gallus gallus*), *Xenopus* (Uniprot entry Q6DFA1; *Xenopus laevis*), fugu (from EST entry CA590467; *Takifugu rubripes*), zebrafish (Uniprot entry A7MD57; *Danio rerio*), shark (from EST entry ES606928; *Squalus acanthias*), sea urchin (from EST entry CX698454; *Strongylocentrotus purpureus*), lancelet (from EST entry BW704568; *Branchiostoma floridae*), Acropora (from EST entry DY582258; *Acropora millepora*), Nematostella (from EST entry FC297726; *Nematostella vectensis*). (*C*) Extracts prepared from HeLa cells harboring an HA-control plasmid (HA) or expressing as indicated HA-tagged DCNL3 or DCNL3-C4A-C8A were treated with NEM to block free cystein residues. Palmitoylated Cys were cleaved with (+) or without NH_2-OH (−, negative control) and exposed Cys were subsequently coupled to biotin-maleimide followed by immunoprecipitation with HA antibodies. Biotinylated DCNL3 was visualized with Avidin-HRP. (*D*) The localization of Cul1 was visualized with specific antibodies in HeLa cells, harboring an HA-control plasmid (HA) or expressing HA-tagged DCNL3, by indirect immunofluorescence. The overlay pictures show DNA staining (DAPI, blue) together with DCNL (HA, red) and Cul (green) staining. (Bars: 5 μm.) (*E*) Analysis of the subcellular localization of Cul3 and its membrane recruitment by immunofluorescence staining in HeLa cells overexpressing HA-tagged wild type or the indicated DCNL3 mutants using HA (red) or Cul3 (green) antibodies. DNA was visualized by DAPI (blue). Arrows indicate the accumulation of Cul3 at plasma membranes. (Bars: 5 μm.)





C

Cul3	Mean ratio inhibitor/control (neddyl/unneddyl)	Standard deviation	t-test
control	1	0	
HMA	0.64	0.08	0.001
2BP	0.67	0.27	0.05

D

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Cdc53                      K760                                  R804
Cdc53       746 ERQIFLEACIVRIMKAKRNLPHTTLVNECIAQSHQRFNAKVSMVKRAIDSLIQKGYLQRGDDG-ESYAYLA  C-term
Cullin 1    706 ERKLLIQAAIVRIMKMRKVLKHQQLLGEVLTQLSSRFKPRVPVIKKCIDILIEKEYLERVDGEKDTYSYLA  C-term
Cullin 2    675 DRKMYLQAAIVRIMKARKVLRHNALIQEVISQSRARFNPSISMIKKCIEVLIDKQYIERSQASADEYSYVA  C-term
Cullin 3    698 DRKHEIEAAIVRIMKSRKKMQHNVLVAEVTQQLKARFLPSPVVIKKRIEGLIEREYLARTPEDRKVYTYVA  C-term
Cullin 4A   591 DRQYQIDAAIVRIMKMRKTLGHNLLVSELYNQLKRPVKPG--DLKKRIESLIDRDYMERDKDNPNQYHYVA  C-term
Cullin 4B   845 DRQYQIDAAIVRIMKMRKTLSHNLLVSEVYNQLKFPVKPA--DLKKRIESLIDRDYMERDKENPNQYNYIA  C-term
Cullin 5    710 LRILRTQEAIIQIMKMRKKISNAQLQTELVEILKNMFLPQKKMIKEQIEWLIEHKYIRRDESDINTFIYMA  C-term
Cullin 3                   K712                                  R756
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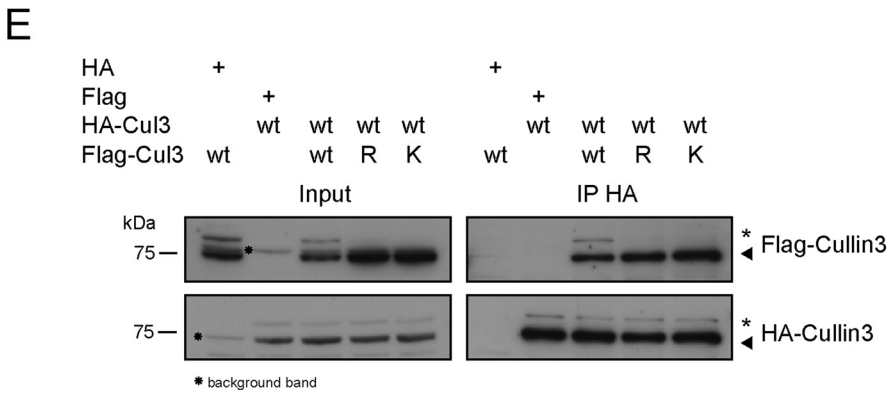
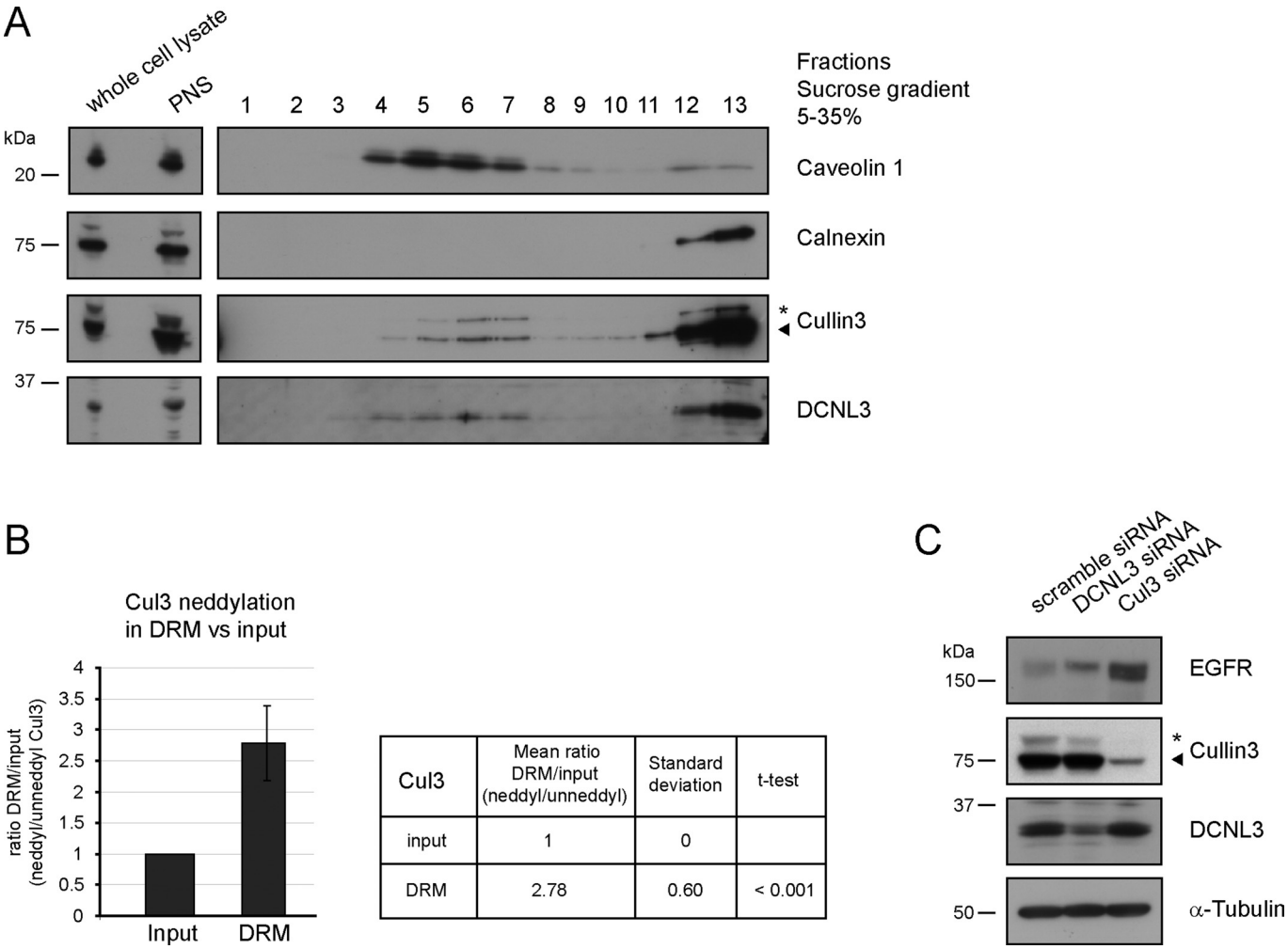


Fig. S6. Characterization of DCNL3 and Cul3 mutants. (*A*) Extracts prepared from HeLa cells transfected with an HA-control plasmid (HA) or plasmids expressing HA-tagged DCNL3 or DCNL3Δ75 (input, *Left*) were incubated with HA antibodies (IP HA). The immunoprecipitates were analyzed for the presence of endogenous Cul3 (*Upper*) and the HA-tagged proteins (*Lower*). (*B*) HeLa cells were transfected with wild-type HA-tagged Cul3 or additionally fused to the N-terminal motif of DCNL3 [amino acids 1–11 of DCNL3, DCNL3 (11)-HA-Cul3]. Cells were analyzed by indirect immunofluorescence with HA antibodies. (Bars: 5 μm.) (*C*) The neddylation state of endogenous Cul3 was quantified in HeLa cells treated with the myristoylation and palmitoylation inhibitors HMA and 2BP as described in Fig. 4*D*. Listed are the mean values and standard deviations of the ratio between cells treated with inhibitor versus DMSO control of neddylated Cul3 over unneddylated Cul3 from 3 independent experiments. Student's *t* test was performed to assess the significance of the difference between DMSO control and inhibitor treatment for the mean ratio. Values <0.01 are judged as highly significant. (*D*) Sequence alignment of the C terminus of yeast Cdc53 with human Culs. Highlighted in gray are the neddylation site K760 in Cdc53 or K712 in Cul3, and the scDcn1 interaction site R804 in Cdc53, which corresponds to R756 in Cul3. (*E*) HeLa lysates (input), transfected with different combinations of HA-Cul3 and Flag-tagged wild-type or mutant Cul3, were immunoprecipitated with HA antibodies (IP HA) and the association to the Flag-tagged mutants analyzed by immunoblotting with specific antibodies. R: Cul3-R756A; K: Cul3-K712R.



Cul3	Mean ratio DRM/input (neddyl/unneddyl)	Standard deviation	t-test
input	1	0	
DRM	2.78	0.60	< 0.001

Fig. S7. Membrane-activated Cul3 may regulate intracellular trafficking pathways. (*A*) HeLa cells were lyzed in 1% Triton X-100 (whole cell) and the postnuclear supernatant (PNS) was loaded under a 5–35% sucrose gradient. DRMs float in the gradient because of their lipid composition and cholesterol content whereas solubilized membranes are pelleted to the bottom. The gradient was harvested from the top (fraction 1: lightest fraction) and each fraction was tested for the presence of Cul3 and DCNL3 by using specific antibodies. Marker proteins for DRMs: Caveolin1; for soluble membranes: Calnexin. (*B*) The ratio of neddylated over unneddylated Cul3 was determined from 3 independent experiments comparing the Cul3 peak fraction in the DRMs to the Cul3 in the input fraction. Student's *t* test was performed to assess the significance of the difference between lysate input and DRM neddylation. Values <0.01 are judged as highly significant. (*C*) Whole-cell lysates of HeLa cells depleted of DCNL3, Cul3, or with a control scramble siRNA oligo were analyzed by immunoblotting with EGFR antibodies. Depletion efficiency was controlled with antibodies specific for DCNL3 or Cul3, while α-tubulin was used to control for equal loading. Note that EGFR accumulates in cells depleted for Cul3 and to a lesser extent upon down-regulation of DCNL3. Arrowhead indicates unneddylated Cul3; asterisk indicates neddylated Cul3.