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# LDL Receptor-related Protein 1 Regulates the Abundance of Diverse Cell-signaling Proteins in the Plasma Membrane Proteome

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## Abstract

LDL receptor-related protein 1 (LRP1) is an endocytic receptor, reported to regulate the abundance of other receptors in the plasma membrane, including uPAR and tissue factor. The goal of this study was to identify novel plasma membrane proteins, involved in cell-signaling, which are regulated by LRP1. Membrane protein ectodomains were prepared from RAW 264.7 cells in which LRP1 was silenced and control cells using protease K. Peptides were identified by LC-MS/MS. By analysis of spectral counts, 31 transmembrane and secreted proteins were regulated in abundance at least 2-fold when LRP1 was silenced. Validation studies confirmed that semaphorin4D (Sema4D), plexin domain-containing protein-1 (Plxdc1), and neuropilin-1 were more abundant in the membranes of LRP1 gene-silenced cells. Regulation of Plxdc1 by LRP1 was confirmed in CHO cells, as a second model system. Plxdc1 co-immunoprecipitated with LRP1 from extracts of RAW 264.7 cells and mouse liver. Although Sema4D did not co-immunoprecipitate with LRP1, the cell-surface level of Sema4D was increased by RAP, which binds to LRP1 and inhibits binding of other ligands. These studies identify Plxdc1, Sema4D, and neuropilin-1 as novel LRP1-regulated cell-signaling proteins. Overall, LRP1 emerges as a generalized regulator of the plasma membrane proteome.

### Keywords

LRP1; Plxdc1; Semaphorin4D; neuropilin-1; plasma membrane

## Introduction

LDL receptor-related protein 1 (LRP1) is an endocytic receptor for diverse ligands, including proteases, protease inhibitors, growth factors, extracellular matrix proteins, and lipoproteins1. In many cell types, including neurons and Schwann cells, binding and endocytosis of ligands by LRP1 triggers robust cell-signaling responses2–6. LRP1 also regulates cell-signaling and the response to extracellular mediators by acting as a co-receptor, a response modifier, and/or by controlling the cell-surface abundance of other receptors, such as the urokinase receptor (uPAR), PDGF receptor-β, TNF receptor-1

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(TNFR1), and toll-like receptor-4[7–11]. By its effects on cell-signaling, LRP1 regulates cell survival, migration, differentiation, inflammation, and vascular permeability[6,8,10,12–17].

Many activities of LRP1 are explained mechanistically by its ability to regulate the abundance of other proteins in the plasma membrane. uPA-Serpin complexes bind simultaneously to uPAR and LRP1 so the entire complex is internalized by LRP1[18]. Similarly, Factor VIIa-tissue factor pathway inhibitor complex bridges tissue factor to LRP1 so that the tissue factor is cleared from the cell surface[19]. LRP1 regulates trafficking and amyloidigenic processing of amyloid precursor protein (APP) by forming a bridged-receptor complex in which Fe65 binds both LRP1 and APP[20,21]. $PrP^c$ binds directly to LRP1 and undergoes LRP1-mediated clearance from the cell surface[22]. Still other membrane proteins, such as uPAR-AP/Endo-180, are regulated by LRP1 at the transcriptional level, apparently downstream of LRP1-dependent cell-signaling[23]. Understanding the activity of LRP1 as a regulator of the plasma membrane proteome remains an important objective, which may help explain activities of LRP1 in prevalent diseases, such as atherogenesis[24], cancer metastasis[25], and stroke[5,17].

The goal of this study was to identify novel plasma membrane proteins that are regulated by LRP1. The use of proteomics to study plasma membrane composition remains challenging. A number of strategies have been developed[26,27]. Our approach was to apply a protease-based method for acquiring and analyzing membrane protein ectodomains, referred to as proteome of cell-exposed extracellular domains (PROCEED), originally described by Bledi et al[28]. RAW 264.7 macrophage-like cells in which LRP1 was silenced and control cells were compared. Thirty-one LRP1-regulated transmembrane and secreted proteins were identified. Validation studies were performed, confirming that three transmembrane proteins are significantly more abundant in macrophage plasma membranes when LRP1 is deficient. The validated proteins were neuropilin-1 (Nrp1), which functions as a co-receptor for class 3 semaphorins and vascular endothelial growth factor, plexin domain-containing protein-1 (Plxdc1), and the membrane-anchored semaphorin, Sema4D.

## Methods

### Reagents

Sulfo-NHS-LC-biotin was from Pierce (Rockford, IL). Papain and protease-K were from Worthington Biochemical (Lakewood, NJ). Nrp-1-specific antibody was from Cell Signaling Technologies (Danvers, MA). Sema4D-specific antibody was from Abcam (Cambridge, MA). Plxdc1-specific antibody was from Imgenex (San Diego, CA). LRP1-specific antibody 11H4 was purchased from ATCC. Receptor-associated protein (RAP) was prepared as previously described[29]. Endotoxin-free RAP was prepared using endotoxin-removing columns (Pierce), according to manufacturer's instructions.

### Cell Culture

RAW 264.7 macrophage-like cells were cultured in RPMI 1640, supplemented with 10 mM L-glutamine, 10% FBS, and penicillin/streptomycin. LRP1-deficient CHO 13-5-1 cells and LRP1-expressing CHO K1 cells were cultured in DMEM low glucose medium, supplemented with 10% FBS (HyClone Laboratories, Logan, UT), non-essential amino acids, 2 mM L-glutamine, 100 units/ ml of penicillin, and 100 μg/ml of streptomycin[30,31].

### LRP1 Gene-silencing in RAW 264.7 Cells

Silencing of LRP1 in RAW 264.7 cells was accomplished using the pSUPER vector system from Oligoengine (Seatlle, WA), which expresses shRNA directed against LRP1 as previously described[25]. RAW 264.7 cells were transfected with this construct or with empty

vector using the Nucleofector system from Amaxa. Transfected cells were selected with puromycin (1 μg/mL). LRP1 gene-silenced cells were treated with *Pseudomonas* exotoxin A for 48 h (250 ng/ml) to eliminate LRP1-positive cells32.

### PROCEED Preparations

LRP1-expressing and gene-silenced RAW 264.7 cells were cultured in serum-containing medium until confluent. Cells were dissociated by gentle scraping, washed with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), and then re-suspended in PBS containing papain or protease K (1/100 w/w) for 1 h at 4 °C. Cells and cell fragments were pelleted by centrifugation. The protease activity was neutralized by addition of phenylmethylsulfonyl fluoride or E64. Proteins in the cell-free medium were precipitated by addition of 20% trichloroacetic acid and after centrifugation, re-suspended in SDS-PAGE loading-buffer. SDS-PAGE was conducted.

### LC-MS/MS and Data Analysis

PROCEED preparations were analyzed as previously described33. After SDS-PAGE, the gel was sliced into 19 sections, 0.5 cm in height. In-gel trypsin digestion was then conducted. The resulting peptides were loaded onto a 100 μm fused silica capillary column containing 10 cm of C18 resin (Phenomenex). Peptides were eluted from the column using a 2 h gradient and a flow rate of 0.25 μL/min, directly into an LTQ-XL ion trap MS (ThermoFisher). The LTQ-XL MS was operated in data-dependent scanning mode, with one full MS scan followed by seven MS/MS scans of the most abundant ions with dynamic exclusion enabled. Raw MS/MS data were analyzed using SEQUEST software33 and the DTASelect search program34. Search criteria were established to maintain a maximum false positive rate of 5% and required identification of at least two peptides per locus. DTAselect results were assembled into peptographs, as previously described33. The complete PROTOMAP dataset is available online and can be interactively searched and sorted at the following url: http://tinyurl.com/lqz63n.

### Surface Protein Biotinylation and Immunoprecipitation

Cell-surface proteins were biotinylated using the membrane-impermeable reagent, sulfo-NHS-LC-biotin, and purified by Streptavidin-Sepharose affinity-precipitation, as previously described8. LRP1 immunoprecipitation with monoclonal antibody 11H4 was performed as described by Barnes et al35. Whole cell extracts were prepared in ice-cold RIPA buffer (PBS with 1% NP-40, 0.1% SDS, and 0.5% deoxycholic acid) containing complete protease inhibitor cocktail (Roche). These various preparations were analyzed by SDS-PAGE and immunoblotting, as previously described8.

## Results

### Isolation of Plasma Membrane Ectodomains from LRP1-expressing and -deficient Cells

To compare the plasma membrane proteome in LRP1-expressing and -deficient cells, we isolated plasma membrane protein ectodomains using protocols based on the original PROCEED method described by Bledi et al.28. As shown in Fig 1A, cells in suspension were treated with broad-spectrum proteases, such as papain or protease K, for 1 h at 4°C. The cells were pelleted by centrifugation and peptides in solution were subjected to SDS-PAGE. Each gel was sliced into nineteen sections and the peptides contained in each section were further digested with trypsin, facilitating identification by LC MS/MS33.

As a model system, we studied RAW 264.7 macrophage-like cells. LRP1 expression in macrophages controls inflammation and atherosclerosis8,24. Constitutive expression of LRP1-specific shRNA induced nearly complete LRP1 gene-silencing in RAW 264.7 cells,

as determined by immunoblot analysis (Fig. 1B). LRP1-expressing (LRP1+) and gene-silenced (LRP1−) RAW 264.7 macrophages were subjected to protease digestion to release plasma membrane protein ectodomains. The Sypro ruby-stained gel in Fig 1C shows recovery of numerous peptides in the medium. The absence of tubulin in the medium confirmed that cells and cell fragments did not substantially contaminate the ectodomain preparations (Fig.1D).

## Analysis of the RAW 264.7 Cell Plasma Membrane Proteome

PROCEED preparations generated with protease K were analyzed by LC MS/MS. In isolates from LRP1-expressing RAW 264.7 cells, peptides from 1281 gene products were identified, 11.4% of which were transmembrane proteins. The fraction of transmembrane proteins was increased 12-fold compared with that observed by analysis of whole cell extracts. In three replicate analyses, 31 proteins with transmembrane domains and/or signal sequences were at least two-fold more or less abundant when LRP1 was silenced (Table 1).

In PROCEED preparations from control RAW 264.7 cells, LRP1-related spectral counts were abundant, probably reflecting the large ectodomain in LRP1 and the high level of LRP1 expression in the RAW 264.7 cell plasma membrane. By analysis of spectral counts, LRP1 was silenced by 93% in shRNA-expressing cells. Two other members of the LRP gene family, Sorl1 and LRP1b, were identified at low abundance in PROCEED preparations from LRP1-expressing RAW 264.7 cells. Sorl1 and LRP1b were absent in preparations from LRP1 gene-silenced cells. This may reflect regulation of these LRP family members by LRP1 or cross-reactivity of our LRP1-specific shRNA. BLAST analysis of the LRP1 shRNA sequence against the sequences of Sorl1 and LRP1b showed 53% and 37% identity, respectively. Serpinb9b was increased in abundance in isolates from LRP1 gene-silenced cells. Multiple Serpins are well-defined LRP1 ligands, which undergo endocytosis and degradation in lysosomes following LRP1-binding1. In the absence of LRP1, Serpins may accumulate at the cell surface. Other gene products that were apparently regulated by LRP1 include myeloid cell-specific pathogen receptors (Mrc1, Clec7a, Man2b2 and Msr) and immunoglobulin receptors (Pira3 and Cd300a). Proteins involved in cell adhesion were identified (Ezr, Cd36, Itgam and Fndc3). Validation studies will be required to further assess these results.

## Regulation of Plxdc1, Nrp1, and Sema4D by LRP1

A peptograph, summarizing the LC-MS/MS results for LRP1, is shown in Fig. 2. Results obtained with LRP1 gene-silenced and -expressing cells are presented in blue and red, respectively. The right-hand panel shows the abundance of LRP1-related peptides in each gel section (mean ± S.D., n=3). Low mobility gel sections contain larger peptides. The left-hand panel shows the relationship of identified peptides to the full-length sequence of LRP1. Note that the majority of the larger peptides are derived from the N-terminal and central region of LRP1, as would be anticipated for this type 1 transmembrane protein.

In this study, we chose to validate proteomics data for three transmembrane proteins (Plxdc1, Sema4D, and Nrp1) implicated in cell-signaling. As shown in Fig. 2, in each case, the proteomics data suggested that the plasma membrane abundance of the protein of interest was increased in LRP1 gene-silenced cells. All of the peptides identified for each of the three gene products were derived from the N-terminus of the protein sequence, supporting the major presupposition of the experimental approach, which is selective isolation of membrane protein ectodomains.

To validate that LRP1 gene-silencing increases the cell surface abundance of Plxdc1, Sema4D, and Nrp1, cell-surface proteins in RAW 264.7 cells were labeled with the

impermeable biotinylation reagent, sulfo-NHS-LC-biotin, and affinity-precipitated with Streptavidin-Sepharose. Affinity precipitates and whole cell extracts were subjected to immunoblot analysis. As shown in Fig. 3, the cell surface abundance of Plxdc1 and Sema4D was substantially increased in LRP1 gene-silenced cells. Total cellular levels of the two proteins were unchanged.

Cell-surface Nrp1 was recovered in two bands, a major band at 80-kDa and a minor band at 110-kDa. Both species were increased in intensity when LRP1 was silenced. The same two bands were observed in whole cell extracts, although the ratio was reversed so that the 110-kDa band was more prominent. The 80-kDa band, in the whole cell extracts, was selectively increased in intensity when LRP1 was silenced, supporting the argument that this band represents a major surface-exposed form of Nrp1 that is regulated by LRP1.

### Analysis of the Mechanism by which LRP1 Regulates Membrane Proteins

LRP1 decreases the cell-surface abundance of uPAR, APP, and tissue factor by facilitating the endocytosis of these receptors. In each case, a bridging protein or protein complex links LRP1 to the second receptor36. To test how LRP1 regulates the cell surface abundance of Plxdc1, Sema4D, or Nrp1, first we performed co-immunoprecipitation experiments. Fig. 4A shows that Plxdc1 co-immunoprecipitated with LRP1 from RAW 264.7 cell extracts. Nrp1 and Sema4D were not detected in the equivalent immunoprecipitates. To confirm these results, LRP1 was immunoprecipitated from extracts of mouse liver. Again, Plxdc1 co-immunoprecipitated with LRP1 (Fig. 4B). These results demonstrate that LRP1 associates with Plxdc1, either directly or indirectly.

As a second model system to test whether LRP1 regulates the cell-surface abundance of Plxdc1, we studied CHO 13-5-1 cells in which LRP1 is absent and wild-type LRP1-expressing CHO K1 cells. This model system is previously described31. As shown in Figure 4C, the cell-surface abundance of Plxdc1 was significantly increased in LRP1-deficient CHO 13-5-1 cells. The total cellular level of Plxdc1 was unchanged. Sema4D and Nrp1 were not detected in CHO cells.

RAP binds to the ligand-binding repeats in LRP1 and inhibits interactions that are critical for LRP1-facilitated membrane protein endocytosis21,36,37. Because not all protein interactions can be detected by co-immunoprecipitation, we cultured LRP1-expressing RAW 264.7 cells for three days in the presence of RAP, as previously described21, 37, and then, determined the cell-surface level of Sema4D. Fig. 4D shows that the cell-surface abundance of Sema4D was substantially increased by RAP, supporting the hypothesis that Sema4D trafficking and subcellular distribution are regulated by LRP1.

## Discussion

LRP1 gene deletion in mouse is embryonic lethal38. Conditional LRP1 gene deletion in vascular smooth muscle cells, macrophages, or neurons generates robust phenotypes, suggesting that LRP1 regulates multiple aspects of cell physiology7,24,39,40. One mechanism by which LRP1 regulates cell physiology is by clearing growth factors and proteases from the extracellular spaces1. In some cell types, binding of LRP1 ligands activates cell-signaling2,4,14. LRP1 also is subject to regulated intramembrane proteolysis; the resulting C-terminal fragment of LRP1 may translocate to the nucleus and regulate gene transcription10,41. LRP1 functions as a co-receptor for PDGF receptor-β11,42. LRP1 also decreases the cell-surface abundance of receptors such as uPAR, APP, tissue factor, and TNFR18,36. This is an important pathway whereby LRP1 may regulate the physiology of diverse cells. To understand the extent to which this pathway contributes to the overall activity of LRP1, it is important to define the LRP1-regulated membrane protein proteome.

In this study, we utilized PROCEED to compare plasma membrane proteomes. This method circumvents challenges resulting from the hydrophobic nature of transmembrane proteins and the low solubility of proteins in domains such as lipid rafts28. Avoiding the use of detergents eliminates technical problems otherwise encountered in sample preparation and analysis26–28. The PROCEED technique has the capacity to identify membrane proteins that are altered in concentration in the plasma membrane. Changes in recovery of peptides from a specific membrane protein also may reflect altered membrane domain localization, association with other proteins in the membrane, and/or conformational heterogeneity influencing susceptibility to proteolytic cleavage. Using a single protease (protease K), multiple candidate LRP1-regulated gene products were identified. Three of the identified candidates were validated. The other targets listed in Table 1 require further study. A number of cellular receptors that are known to be regulated by LRP1, including TNFR1 and uPAR8,18, were not identified in the preparations characterized here. Thus, the membrane proteins identified in this study represent a subset of those that are regulated by LRP1. Additional regulated membrane proteins may be identified using different proteases, such as papain, or by altering the PROCEED conditions.

Using biotin-labeling and affinity precipitation, we validated Sema4D, Plxdc1 and Nrp1 as LRP1-regulated plasma membrane proteins. The plasma membrane abundance of these proteins was increased when LRP1 was silenced in RAW 264.7 macrophage-like cells. Plxdc1 also was increased at the cell surface in LRP1-deficient CHO cells. The levels of Sema4D and Plxdc1 in whole cell extracts were unchanged, suggesting that LRP1 regulates the subcellular distribution of these receptors by facilitating their endocytosis. It is well known that membrane proteins that associate with LRP1 undergo endocytosis with LRP1 in clathrin-coated pits36. We showed that Plxdc1 associates with LRP1 by co-immunoprecipitation. Although we were not able to demonstrate co-immunoprecipitation of Nrp1 and Sema4D with LRP1, the cell surface abundance of Sema4D was increased in cells treated with RAP. Culturing cells in the presence of RAP neutralizes interactions involving LRP1 and other membrane proteins that are frequently necessary for LRP1-facilitated endocytosis21,36,37. Overall, these results indicate that the ability of LRP1 to promote endocytosis of membrane proteins and facilitate their clearance from the cell surface may be more ubiquitous than previously appreciated.

The semaphorins and their receptors in the plexin family and Nrp1 were originally identified as guidance factors that function in axonal development and synapse formation43. However, more recent studies have identified a wide spectrum of activities for these proteins in diverse biological processes, including heart morphogenesis, blood vessel formation, and immune cell regulation44–46. Sema4D is involved in B cell differentiation and T cell activation47,48 and is a potent pro-angiogenic factor in cancer49,50. The source of Sema4D, in tumor angiogenesis, is most likely macrophages49. Sema4D also is expressed by platelets and may function as a prothrombotic membrane protein51.

Plxdc1 is also known as tumor endothelial marker-7 and is expressed mainly by endothelial cells in tumors52–54. Although the function of Plxdc1 remains incompletely understood, it may serve as a cell-signaling receptor for nidogen55. Nrp1 functions as a co-receptor for class 3 semaphorins and for vascular endothelial growth factor56,57. Nrp1 has been implicated in cancer progression57. Nrp1 is known to associate with β1 integrins and with c-Met in the plasma membrane58,59. In macrophages, expression of Nrp1 is increased by colony-stimulating factor-160, as is LRP161.

Like the semaphorins, plexins, and Nrp1, LRP1 has been implicated in axonal growth, synapse function, and long-term potentiation4,39. We previously demonstrated that in cultured hippocampal neurons, LRP1 localizes in a polarized manner to cell bodies and

dendritic spines and shafts62. Others have shown that LRP1 binds post-synaptic density protein-95 (PSD-95), a scaffolding protein that bridges multiple proteins involved in signaling responses in dendritic spines39,63,64. It will be important to determine whether the regulatory activities of LRP1 demonstrated here are operational in neurons and whether these activities contribute to the role of LRP1 in complex processes such as long term potentiation.

## Abbreviations

| | |
|---|---|
| **LRP1** | LDL receptor-related protein-1 |
| **uPAR** | urokinase receptor |
| **APP** | amyloid precursor protein |
| **TNFR1** | tumor necrosis factor receptor-1 |
| **PROCEED** | proteome of cell-exposed extracellular domains |
| **Nrp1** | neuropilin-1 |
| **plxdc1** | plexin domain-containing protein-1 |
| **Sema4D** | semaphorin4D |
| **RAP** | receptor-associated protein |

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## References

1. Strickland DK, Gonias SL, Argraves WS. Diverse roles for the LDL receptor family. Trends Endocrinol Metab. 2002; 13(2):66–74. [PubMed: 11854021]
2. Mantuano E, Inoue G, Li X, Takahashi K, Gaultier A, Gonias SL, Campana WM. The hemopexin domain of matrix metalloproteinase-9 activates cell signaling and promotes migration of schwann cells by binding to low-density lipoprotein receptor-related protein. J Neurosci. 2008; 28(45): 11571–11582. [PubMed: 18987193]
3. Qiu Z, Hyman B, Rebeck G. Apolipoprotein E receptors mediate neurite outgrowth through activation of p44/42 mitogen-activated protein kinase in primary neurons. J Biol Chem. 2004; 279(33):34948–34956. [PubMed: 15169786]
4. Shi Y, Mantuano E, Inoue G, Campana WM, Gonias SL. Ligand binding to LRP1 transactivates Trk receptors by a Src family kinase-dependent pathway. Sci Signal. 2009; 2(68):ra18. [PubMed: 19401592]
5. Zhang X, Polavarapu R, She H, Mao Z, Yepes M. Tissue-type plasminogen activator and the low-density lipoprotein receptor-related protein mediate cerebral ischemia-induced nuclear factor-kappaB pathway activation. Am J Pathol. 2007; 171(4):1281–1290. [PubMed: 17717150]
6. Zhuo M, Holtzman DM, Li Y, Osaka H, DeMaro J, Jacquin M, Bu G. Role of tissue plasminogen activator receptor LRP in hippocampal long-term potentiation. J Neurosci. 2000; 20(2):542–549. [PubMed: 10632583]
7. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: role in vascular wall integrity and protection from atherosclerosis. Science. 2003; 300(5617):329–332. [PubMed: 12690199]
8. Gaultier A, Arandjelovic S, Niessen S, Overton CD, Linton MF, Fazio S, Campana WM, Cravatt BF 3rd, Gonias SL. Regulation of tumor necrosis factor receptor-1 and the IKK-NF-kappaB pathway by LDL receptor-related protein explains the antiinflammatory activity of this receptor. Blood. 2008; 111(11):5316–5325. [PubMed: 18369152]

9. Webb D, Thomas K, Gonias S. Plasminogen activator inhibitor 1 functions as a urokinase response modifier at the level of cell signaling and thereby promotes MCF-7 cell growth. J Cell Biol. 2001; 152(4):741–752. [PubMed: 11266465]

10. Zurhove K, Nakajima C, Herz J, Bock HH, May P. Gamma-secretase limits the inflammatory response through the processing of LRP1. Sci Signal. 2008; 1(47):ra15. [PubMed: 19036715]

11. Muratoglu SC, Mikhailenko I, Newton C, Migliorini M, Strickland DK. The LDL receptor-related protein 1 (LRP1) forms a signaling complex with the PDGF receptor-{beta} in endosomes and regulates activation of the mapk pathway. J Biol Chem. 2010

12. Campana WM, Li X, Dragojlovic N, Janes J, Gaultier A, Gonias SL. The low-density lipoprotein receptor-related protein is a pro-survival receptor in Schwann cells: possible implications in peripheral nerve injury. J Neurosci. 2006; 26(43):11197–11207. [PubMed: 17065459]

13. Hayashi H, Campenot RB, Vance DE, Vance JE. Apolipoprotein E-containing lipoproteins protect neurons from apoptosis via a signaling pathway involving low-density lipoprotein receptor-related protein-1. J Neurosci. 2007; 27(8):1933–1941. [PubMed: 17314289]

14. Hu K, Yang J, Tanaka S, Gonias SL, Mars WM, Liu Y. Tissue-type Plasminogen Activator Acts as a Cytokine That Triggers Intracellular Signal Transduction and Induces Matrix Metalloproteinase-9 Gene Expression. J Biol Chem. 2006; 281(4):2120–2127. [PubMed: 16303771]

15. Ma Z, Thomas KS, Webb DJ, Moravec R, Salicioni AM, Mars WM, Gonias SL. Regulation of Rac1 activation by the low density lipoprotein receptor-related protein. J Cell Biol. 2002; 159(6): 1061–1070. [PubMed: 12499359]

16. Qiu Z, Strickland DK, Hyman BT, Rebeck GW. alpha 2-Macroglobulin exposure reduces calcium responses to N-methyl-D-aspartate via low density lipoprotein receptor-related protein in cultured hippocampal neurons. J Biol Chem. 2002; 277(17):14458–14466. [PubMed: 11839752]

17. Yepes M, Sandkvist M, Moore EG, Bugge TH, Strickland DK, Lawrence DA. Tissue-type plasminogen activator induces opening of the blood-brain barrier via the LDL receptor-related protein. J Clin Invest. 2003; 112(10):1533–1540. [PubMed: 14617754]

18. Conese M, Nykjaer A, Petersen CM, Cremona O, Pardi R, Andreasen PA, Gliemann J, Christensen EI, Blasi F. alpha-2 Macroglobulin receptor/Ldl receptor-related protein(Lrp)-dependent internalization of the urokinase receptor. J Cell Biol. 1995; 131(6 Pt 1):1609–1622. [PubMed: 8522616]

19. Hamik A, Setiadi H, Bu G, McEver RP, Morrissey JH. Down-regulation of monocyte tissue factor mediated by tissue factor pathway inhibitor and the low density lipoprotein receptor-related protein. J Biol Chem. 1999; 274(8):4962–4969. [PubMed: 9988740]

20. Pietrzik CYIS, Jaeger S, Busse T, Weggen S, Koo E. FE65 constitutes the functional link between the low-density lipoprotein receptor-related protein and the amyloid precursor protein. J Neurosci. 2004; 24(17):4259–4265. [PubMed: 15115822]

21. Ulery PG, Beers J, Mikhailenko I, Tanzi RE, Rebeck GW, Hyman BT, Strickland DK. Modulation of beta-amyloid precursor protein processing by the low density lipoprotein receptor-related protein (LRP). Evidence that LRP contributes to the pathogenesis of Alzheimer's disease. J Biol Chem. 2000; 275(10):7410–7415. [PubMed: 10702315]

22. Parkyn CJ, Vermeulen EG, Mootoosamy RC, Sunyach C, Jacobsen C, Oxvig C, Moestrup S, Liu Q, Bu G, Jen A, Morris RJ. LRP1 controls biosynthetic and endocytic trafficking of neuronal prion protein. J Cell Sci. 2008; 121(Pt 6):773–783. [PubMed: 18285446]

23. Gaultier A, Salicioni AM, Arandjelovic S, Gonias SL. Regulation of the composition of the extracellular matrix by low density lipoprotein receptor-related protein-1: activities based on regulation of mRNA expression. J Biol Chem. 2006; 281(11):7332–7340. [PubMed: 16407289]

24. Overton CD, Yancey PG, Major AS, Linton MF, Fazio S. Deletion of macrophage LDL receptor-related protein increases atherogenesis in the mouse. Circ Res. 2007; 100(5):670–677. [PubMed: 17303763]

25. Montel V, Gaultier A, Lester RD, Campana WM, Gonias SL. The low-density lipoprotein receptor-related protein regulates cancer cell survival and metastasis development. Cancer Res. 2007; 67(20):9817–9824. [PubMed: 17942912]

26. Cordwell S, Thingholm T. Technologies for plasma membrane proteomics. Proteomics. 2009

27. Wu C, Yates J. The application of mass spectrometry to membrane proteomics. Nat Biotechnol. 2003; 21(3):2627.
28. Bledi Y, Inberg A, Linial M. PROCEED: A proteomic method for analysing plasma membrane proteins in living mammalian cells. Brief Funct Genomic Proteomic. 2003; 2(3):254–265. [PubMed: 15239928]
29. Gaultier A, Wu X, Le Moan N, Takimoto S, Mukandala G, Akassoglou K, Campana WM, Gonias SL. Low-density lipoprotein receptor-related protein 1 is an essential receptor for myelin phagocytosis. J Cell Sci. 2009; 122(Pt 8):1155–1162. [PubMed: 19299462]
30. Salicioni AM, Gaultier A, Brownlee C, Cheezum MK, Gonias SL. Low density lipoprotein receptor-related protein-1 promotes beta1 integrin maturation and transport to the cell surface. J Biol Chem. 2004; 279(11):10005–10012. [PubMed: 14699139]
31. FitzGerald DJ, Fryling CM, Zdanovsky A, Saelinger CB, Kounnas M, Winkles JA, Strickland D, Leppla S. Pseudomonas exotoxin-mediated selection yields cells with altered expression of low-density lipoprotein receptor-related protein. J Cell Biol. 1995; 129(6):1533–1541. [PubMed: 7790352]
32. Hussaini IM, Brown MD, Weaver AM, Carpenter J, Karns LR, Vandenberg SR, Gonias SL. Stable antisense RNA expression neutralizes the activity of low-density lipoprotein receptor-related protein and promotes urokinase accumulation in the medium of an astrocytic tumor cell line. Antisense Nucleic Acid Drug Dev. 1999; 9(2):183–190. [PubMed: 10355824]
33. Dix MM, Simon GM, Cravatt BF. Global mapping of the topography and magnitude of proteolytic events in apoptosis. Cell. 2008; 134(4):679–691. [PubMed: 18724940]
34. Tabb DL, McDonald WH, Yates JR 3rd. DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. J Proteome Res. 2002; 1(1):21–26. [PubMed: 12643522]
35. Barnes H, Larsen B, Tyers M, van Der Geer P. Tyrosine-phosphorylated low density lipoprotein receptor-related protein 1 (Lrp1) associates with the adaptor protein SHC in SRC-transformed cells. J Biol Chem. 2001; 276(22):19119–19125. [PubMed: 11259429]
36. Gonias SL, Wu L, Salicioni AM. Low density lipoprotein receptor-related protein: regulation of the plasma membrane proteome. Thromb Haemost. 2004; 91(6):1056–1064. [PubMed: 15175790]
37. Webb DJ, Nguyen DH, Sankovic M, Gonias SL. The very low density lipoprotein receptor regulates urokinase receptor catabolism and breast cancer cell motility in vitro. J Biol Chem. 1999; 274(11):7412–7420. [PubMed: 10066806]
38. Herz J, Clouthier DE, Hammer RE. LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. Cell. 1992; 71(3):411–421. [PubMed: 1423604]
39. May P, Rohlmann A, Bock HH, Zurhove K, Marth JD, Schomburg ED, Noebels JL, Beffert U, Sweatt JD, Weeber EJ, Herz J. Neuronal LRP1 functionally associates with postsynaptic proteins and is required for normal motor function in mice. Mol Cell Biol. 2004; 24(20):8872–8883. [PubMed: 15456862]
40. May P, Woldt E, Matz RL, Boucher P. The LDL receptor-related protein (LRP) family: an old family of proteins with new physiological functions. Ann Med. 2007; 39(3):219–228. [PubMed: 17457719]
41. May P, Reddy YK, Herz J. Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. J Biol Chem. 2002; 277(21):18736–18743. [PubMed: 11907044]
42. Boucher P, Liu P, Gotthardt M, Hiesberger T, Anderson RG, Herz J. Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low Density lipoprotein receptor-related protein in caveolae. J Biol Chem. 2002; 277(18):15507–15513. [PubMed: 11854295]
43. Kolodkin AL, Matthes DJ, Goodman CS. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell. 1993; 75(7):1389–1399. [PubMed: 8269517]

44. Behar O, Golden JA, Mashimo H, Schoen FJ, Fishman MC. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. Nature. 1996; 383(6600):525–528. [PubMed: 8849723]
45. Suzuki K, Kumanogoh A, Kikutani H. Semaphorins and their receptors in immune cell interactions. Nat Immunol. 2008; 9(1):17–23. [PubMed: 18087252]
46. Serini G, Valdembri D, Zanivan S, Morterra G, Burkhardt C, Caccavari F, Zammataro L, Primo L, Tamagnone L, Logan M, Tessier-Lavigne M, Taniguchi M, Puschel AW, Bussolino F. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature. 2003; 424(6947):391–397. [PubMed: 12879061]
47. Shi W, Kumanogoh A, Watanabe C, Uchida J, Wang X, Yasui T, Yukawa K, Ikawa M, Okabe M, Parnes JR, Yoshida K, Kikutani H. The class IV semaphorin CD100 plays nonredundant roles in the immune system: defective B and T cell activation in CD100-deficient mice. Immunity. 2000; 13(5):633–642. [PubMed: 11114376]
48. Hall KT, Boumsell L, Schultze JL, Boussiotis VA, Dorfman DM, Cardoso AA, Bensussan A, Nadler LM, Freeman GJ. Human CD100, a novel leukocyte semaphorin that promotes B-cell aggregation and differentiation. Proc Natl Acad Sci U S A. 1996; 93(21):11780–11785. [PubMed: 8876214]
49. Sierra JR, Corso S, Caione L, Cepero V, Conrotto P, Cignetti A, Piacibello W, Kumanogoh A, Kikutani H, Comoglio PM, Tamagnone L, Giordano S. Tumor angiogenesis and progression are enhanced by Sema4D produced by tumor-associated macrophages. J Exp Med. 2008; 205(7): 1673–1685. [PubMed: 18559453]
50. Sun Q, Zhou H, Binmadi NO, Basile JR. Hypoxia-inducible factor-1-mediated regulation of semaphorin 4D affects tumor growth and vascularity. J Biol Chem. 2009; 284(46):32066–32074. [PubMed: 19762474]
51. Zhu L, Bergmeier W, Wu J, Jiang H, Stalker TJ, Cieslak M, Fan R, Boumsell L, Kumanogoh A, Kikutani H, Tamagnone L, Wagner DD, Milla ME, Brass LF. Regulated surface expression and shedding support a dual role for semaphorin 4D in platelet responses to vascular injury. Proc Natl Acad Sci U S A. 2007; 104(5):1621–1626. [PubMed: 17244710]
52. Lee HK, Bae HR, Park HK, Seo IA, Lee EY, Suh DJ, Park HT. Cloning, characterization and neuronal expression profiles of tumor endothelial marker 7 in the rat brain. Brain Res Mol Brain Res. 2005; 136(1–2):189–198. [PubMed: 15893603]
53. St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW. Genes expressed in human tumor endothelium. Science. 2000; 289(5482):1197–1202. [PubMed: 10947988]
54. Beaty RM, Edwards JB, Boon K, Siu IM, Conway JE, Riggins GJ. PLXDC1 (TEM7) is identified in a genome-wide expression screen of glioblastoma endothelium. J Neurooncol. 2007; 81(3):241–248. [PubMed: 17031559]
55. Lee HK, Seo IA, Park HK, Park YM, Ahn KJ, Yoo YH, Park HT. Nidogen is a prosurvival and promigratory factor for adult Schwann cells. J Neurochem. 2007; 102(3):686–698. [PubMed: 17437540]
56. Halloran MC, Wolman MA. Repulsion or adhesion: receptors make the call. Curr Opin Cell Biol. 2006; 18(5):533–540. [PubMed: 16930978]
57. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell. 1998; 92(6):735–745. [PubMed: 9529250]
58. Fukasawa M, Matsushita A, Korc M. Neuropilin-1 interacts with integrin beta1 and modulates pancreatic cancer cell growth, survival and invasion. Cancer Biol Ther. 2007; 6(8):1173–1180. [PubMed: 17726369]
59. Matsushita A, Gotze T, Korc M. Hepatocyte growth factor-mediated cell invasion in pancreatic cancer cells is dependent on neuropilin-1. Cancer Res. 2007; 67(21):10309–10316. [PubMed: 17974973]
60. Ji JD, Park-Min KH, Ivashkiv LB. Expression and function of semaphorin 3A and its receptors in human monocyte-derived macrophages. Hum Immunol. 2009; 70(4):211–217. [PubMed: 19480842]

61. Hussaini IM, Srikumar K, Quesenberry PJ, Gonias SL. Colony-stimulating factor-1 modulates alpha 2-macroglobulin receptor expression in murine bone marrow macrophages. J Biol Chem. 1990; 265(32):19441–19446. [PubMed: 1700978]

62. Brown MD, Banker GA, Hussaini IM, Gonias SL, VandenBerg SR. Low density lipoprotein receptor-related protein is expressed early and becomes restricted to a somatodendritic domain during neuronal differentiation in culture. Brain Res. 1997; 747(2):313–317. [PubMed: 9046007]

63. Feng W, Zhang M. Organization and dynamics of PDZ-domain-related supramodules in the postsynaptic density. Nat Rev Neurosci. 2009; 10(2):87–99. [PubMed: 19153575]

64. Gotthardt M, Trommsdorff M, Nevitt MF, Shelton J, Richardson JA, Stockinger W, Nimpf J, Herz J. Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. J Biol Chem. 2000; 275(33):25616–25624. [PubMed: 10827173]

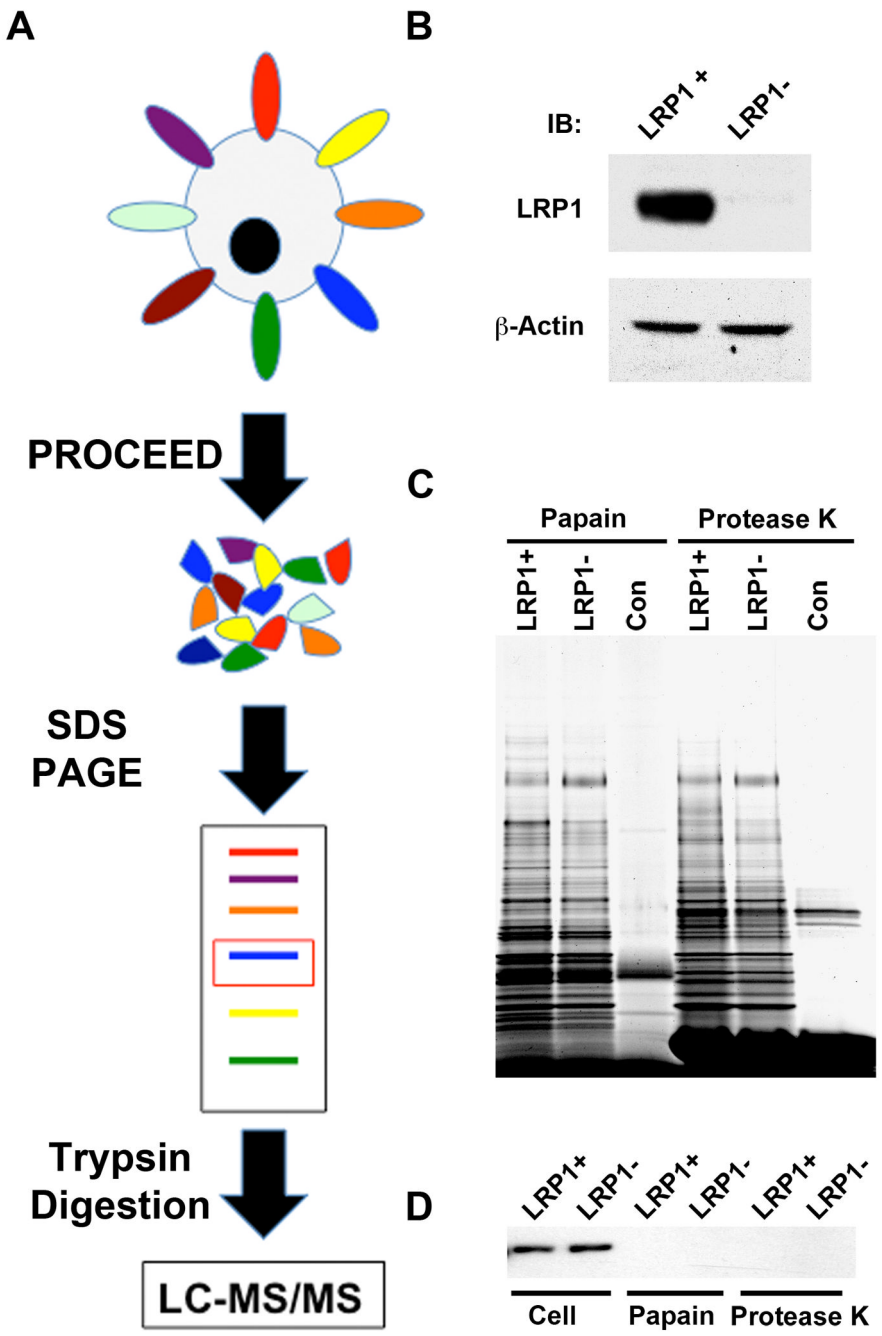


**Figure 1.**

Strategy for generating PROCEED preparations. *A*, Schematic representation of the experimental design. *B*, Whole cell extracts were isolated from LRP1-expressing RAW 264.7 cells (LRP1+) and from cells in which LRP1 was silenced (LRP1−). The extracts were subjected to immunoblot analysis for LRP1 and β-actin. *C*, LRP1+ and LRP1− RAW 264.7 cells were treated with papain or protease K for 1 h. The cells were pelleted and the supernatants were subjected to SDS-PAGE and Sypro ruby-staining. Control lanes (con) show the proteases in the absence of cell supernatants. *D*, PROCEED preparations, obtained using papain or proteinase K, and cell extracts (cell) were subjected to immunoblot analysis for tubulin.

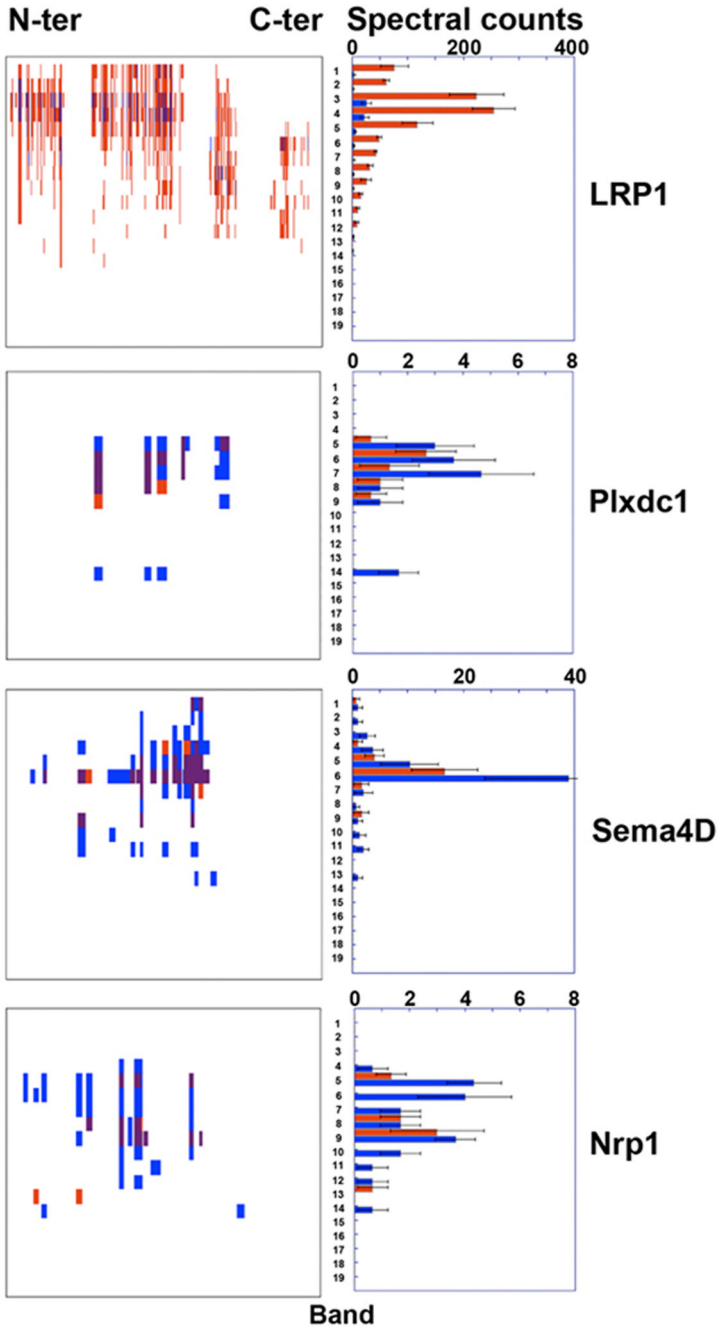


**Figure 2.**

Peptographs for LRP1, Sema4D, Nrp1, and Plxdc1. The y-axis of each peptograph is labeled "1–19" at the center, representing each of the 19 gel slices. The left-hand panel shows the peptides recovered in relation to the sequence of the full-length protein. The right-hand panel shows the spectral counts of peptides related to the gene of interest in each gel section. Results for control LRP1-expressing RAW 264.7 cells are shown in red. Results for cells in which LRP1 is silenced are shown in blue.

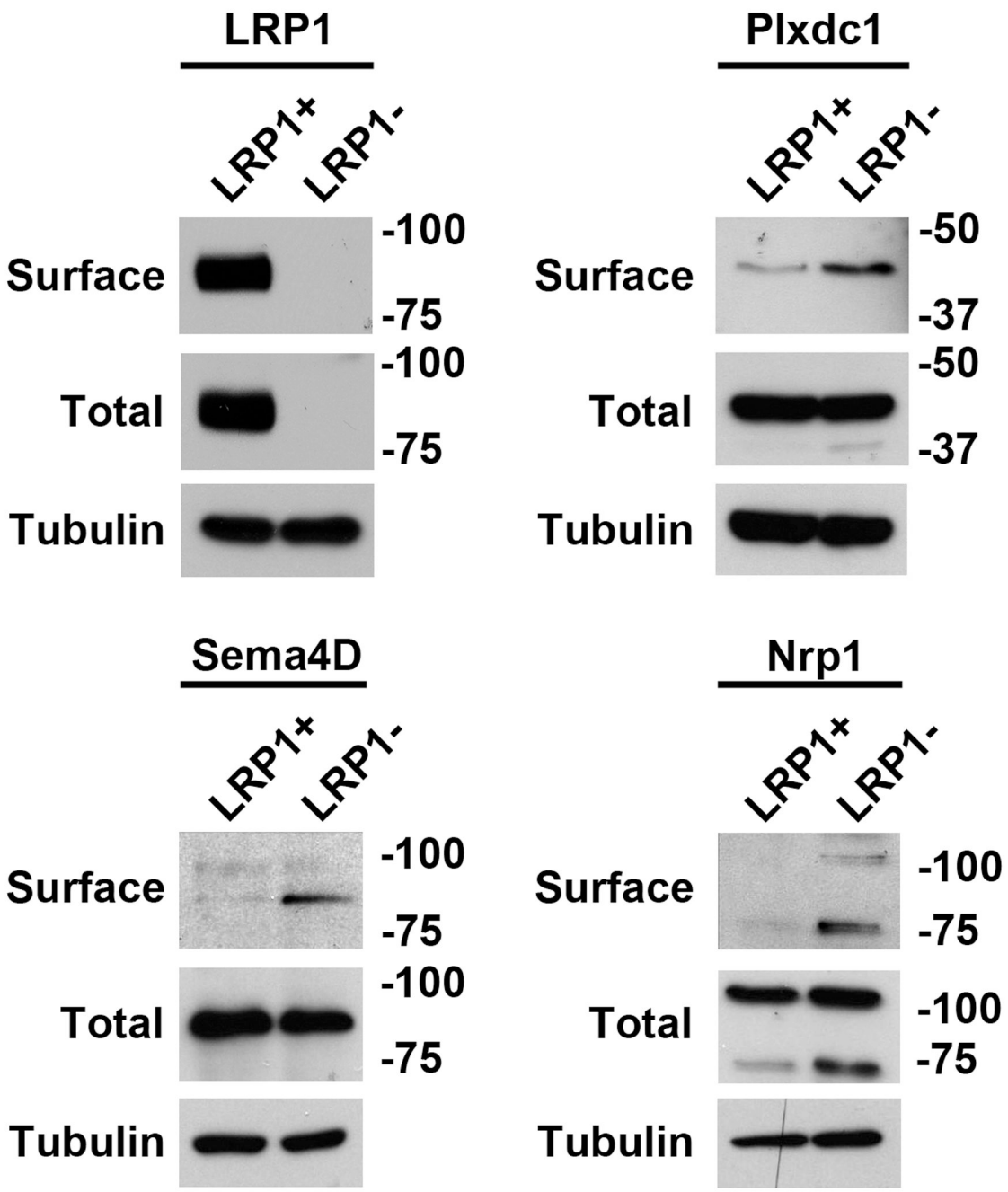


**Figure 3.**

Validation of Sema4D, Nrp1, and Plxdc1 as LRP1-regulated membrane proteins. Cell-surface proteins in control (LRP1+) and LRP1 gene-silenced (LRP1−) RAW 264.7 cells were labeled with biotin, Streptavidin affinity-precipitated, and subjected to immunoblot analysis (surface). Whole cell extracts also were subjected to immunoblot analysis (total). Tubulin was detected in whole cell extracts as a loading control.

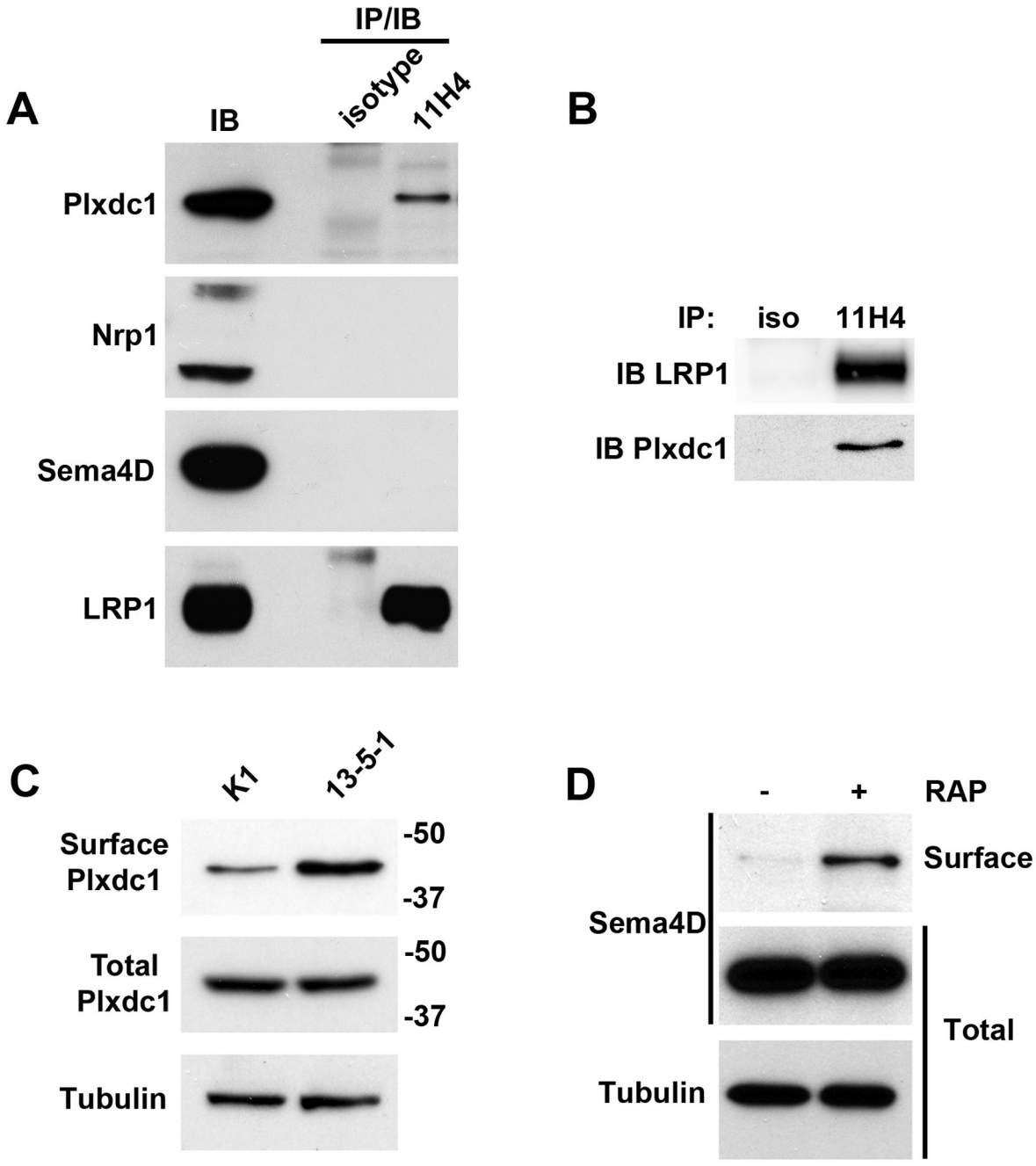


**Figure 4.**

LRP1 associates with Plxdc1 and regulates Sema4D. *A*, Whole cell extracts were isolated from RAW 264.7 cells. Immunoprecipitation was performed using LRP1-specific antibody 11H4 or isotype-matched antibody. The immunoprecipitates (IP) were subjected to immunoblot analysis (IB) to detect LRP1, Sema4D, Nrp1 and Plxdc1. Whole cell extracts were subjected to immunoblot analysis (IB) as a control (left-hand lanes). *B*, The identical immunoprecipitation protocol was executed using mouse liver extracts. Immunoblot analysis was performed to detect LRP1 and Plxdc1. *C*, Cell-surface proteins in control (CHO-K1) and LRP1-deficient (13-5-1) CHO cells were labeled with biotin, Streptavidin affinity-precipitated, and subjected to immunoblot analysis (surface) to detect Plxdc1.

Whole cell extracts also were subjected to immunoblot analysis to detect Plxdc1 (total). Tubulin was detected in whole cell extracts as a loading control. *D*, RAW 264.7 cells were treated with RAP (200 nM) or vehicle for 3 days. Cell-surface Sema4D was determined by biotin-labeling and affinity precipitation. Total levels of Sema4D were determined by analyzing whole cell extracts. Tubulin in whole cell extracts also was determined as a loading control.

## TABLE 1

Plasma membrane and secreted proteins regulated by LRP1 in RAW 264.7 cells.

| Gene name | IPI number | Spectral Counts | | Fold change |
|---|---|---|---|---|
| | | LRP1+ | LRP1− | |
| **Sorl1** | IPI00776230.1 | 8 | 0 | - |
| **Lrp1b** | IPI00119787.4 | 4 | 0 | - |
| **Pira3** | IPI00670237.5 | 5 | 0 | - |
| **Lrp1** | IPI00119063.2 | 917 | 66 | 13.9 |
| **Mrc1** | IPI00126186.1 | 12 | 4 | 3 |
| **Clec7a** | IPI00119904.5 | 4 | 1 | 4 |
| **Fndc3a** | IPI00356888.4 | 6 | 2 | 3 |
| **Man2b2** | IPI00117842.2 | 3 | 1 | 3 |
| **Erap1** | IPI00556880.1 | 3 | 1 | 3 |
| **Itgam** | IPI00120674.2 | 20 | 8 | 2.5 |
| **Cd300a** | IPI00229340.5 | 5 | 2 | 2.5 |
| **Sema4a** | IPI00875256.1 | 21 | 9 | 2.3 |
| **Cd36** | IPI00331214.5 | 3 | 1 | 3 |
| **Strn** | IPI00554884.1 | 4 | 2 | 2 |
| **Msr1** | IPI00128178.1 | 13 | 6 | 2.16 |
| **Plxnb2** | IPI00405742.6 | 35 | 17 | 2.05 |
| **Plxdc1** | IPI00828399.1 | 6 | 15 | 0.4 |
| **Sema4d** | IPI00454115.2 | 26 | 66 | 0.39 |
| **Bst1** | IPI00132279.1 | 4 | 12 | 0.33 |
| **Nrp1** | IPI00123996.1 | 7 | 20 | 0.35 |
| **Fcrl1** | IPI00227259.1 | 2 | 6 | 0.33 |
| **Ezr** | IPI00330862.5 | 4 | 11 | 0.36 |
| **Plxnd1** | IPI00471022.3 | 3 | 9 | 0.33 |
| **Ehd4** | IPI00318671.2 | 1 | 5 | 0.2 |
| **Hyou1** | IPI00123342.4 | 1 | 7 | 0.14 |
| **Lphn2** | IPI00876558.1 | 3 | 16 | 0.19 |
| **Dcbld2** | IPI00131452.1 | 1 | 5 | 0.2 |
| **Dpep2** | IPI00275123.3 | 1 | 6 | 0.12 |
| **Serpinb9b** | IPI00119079.6 | 1 | 8 | 0.08 |
| **Cd47** | IPI00875877.1 | 0 | 4 | 0 |
| **Apcs** | IPI00309214.1 | 0 | 4 | 0 |

The gene products listed below were altered in abundance by LRP1 gene-silencing at least two-fold in PROCEED preparations, as determined by summing total spectral counts in the 19 gel slices. Mean spectral counts are shown for LRP1-positive (LRP+) and gene-silenced (LRP1−) cells (n=3).