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Nuclear translocation and signalling of L1-CAM in human carcinoma cells requires ADAM10 and presenilin/γ-secretase activity

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L1-CAM (L1 cell-adhesion molecule), or more simply L1, plays an important role in the progression of human carcinoma. Overexpression promotes tumour-cell invasion and motility, growth in nude mice and tumour metastasis. It is feasible that L1-dependent signalling contributes to these effects. However, little is known about its mechanism in tumour cells. We reported previously that L1 is cleaved by ADAM (a disintegrin and metalloprotease) and that the cytoplasmic part is essential for L1 function. Here we analysed more closely the role of proteolytic cleavage in L1-mediated nuclear signalling. Using OVMz carcinoma cells and L1-transfected cells as a model, we found that ADAM10-mediated cleavage of L1 proceeds in lipid raft and non-raft domains. The cleavage product, L1-32, is further processed by PS (presenilin)/γ-secretase to release L1-ICD, an L1 intracellular domain of 28 kDa. Overexpression of dominant-negative PS1 or use of a specific γ-secretase inhibitor leads to an accumulation of L1-32. Fluorescence and biochemical analysis revealed a nuclear localization for L1-ICD. Moreover, inhibition of ADAM10 and/or γ-secretase blocks nuclear translocation of L1-ICD and L1-dependent gene regulation. Overexpression of recombinant L1-ICD mediates gene regulation in a similar manner to full-length L1. Our results establish for the first time that regulated proteolytic processing by ADAM10 and PS/γ-secretase is essential for the nuclear signalling of L1 in human carcinoma cell lines.

Key words: a disintegrin and metalloprotease 10 (ADAM10), L1 cell-adhesion molecule (L1-CAM), nuclear translocation, presenilin (PS)/γ-secretase activity, raft, signalling.

INTRODUCTION

L1-CAM (L1 cell-adhesion molecule), or more simply L1, is a 200–220-kDa transmembrane glycoprotein of the immunoglobulin superfamily. Initially, it was found to be involved in the regulation of cell migration, axon outgrowth and guidance during the development of the nervous system [1]. However, more recent studies have shown that L1 also plays a role in the ontogeny of human tumours and its expression is linked to poor prognosis [2–5]. The mechanism by which L1 contributes to tumour progression is not clearly established.

Previous studies have demonstrated that L1 enhances growth of tumour cells in NOD/SCID (non-obese diabetic severe combined immunodeficiency) mice, augments tumour cell motility on extracellular-matrix proteins and invasiveness in matrigel assays, and promotes formation of liver metastases [6,7]. Interference with L1 expression by genetic manipulation was found to be growth inhibitory *in vitro* [8]. Similar results were reported for L1 antibodies [8,9]. Importantly, a number of studies have demonstrated that L1 can alter gene expression [7–9]. Although ERK (extracellular-signal-regulated kinase) activation appears to be required for this process, it is unclear if additional factors are involved.

We demonstrated previously that the ectodomain of L1 is cleaved at the plasma membrane by ADAM10 (A Disintegrin And Metalloprotease 10) [6,10,11]. The involvement of ADAM10 was confirmed in a study using ADAM-deficient fibroblastic cell lines established from knock-out mice [12]. This investigation suggested that ectodomain cleavage by ADAM10 is followed by intramembrane PS (presenilin)/γ-secretase-dependent cleavage, leading to the generation of L1-ICD (L1 intracellular domain) [12]. The process of RIP (regulated intramembrane proteolysis) is an essential step in a variety of signalling pathways [13]. Nuclear translocation and transcriptional regulation of proteins such as Notch, CD44 and APP (amyloid precursor protein) were shown to depend on ADAM-mediated cleavage followed by PS/γ-secretase activity [13]. We thus hypothesized that proteolytic processing might contribute to L1-signalling.

Lipid rafts are microdomains within the plasma membrane that are enriched in cholesterol and sphingolipids [14]. They have gained attention as platforms for the proteolytic processing of several proteins, including APP [15,16] and the cellular prion protein (PrP^c) [17]. Interestingly, it has already been shown that members of the L1 family are associated with cholesterol-enriched microdomains [18,19].

Abbreviations used: ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; CHO, Chinese-hamster ovary; CRABPII, cellular retinoic acid-binding protein II; CTF, C-terminal fragment; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine t-butyl ester (also known as presenilin inhibitor IX); DRM, detergent-resistant membrane; ECL®, enhanced chemiluminescence; ERK, extracellular-signal-regulated kinase; FCS, fetal-calf serum; FNIII, fibronectin III; HEK-293, human embryonic kidney-293; hL1, human L1; HRP, horseradish peroxidase; ICD, intracellular domain; LAMP-1, lysosomal-associated membrane protein 1; L1, L1 cell-adhesion molecule; L1cyt, the cytoplasmic part of L1; mAb, monoclonal antibody; MDK, midkine or neurite growth-promoting factor 2; MT1-MMP, membrane type-1 matrix metalloproteinase; pAb, polyclonal antibody; PS(1), presenilin(-1); qRT-PCR, quantitative real-time PCR; RIP, regulated intramembrane proteolysis; siRNA, small interfering RNA; TACE, tumour-necrosis factor-α converting enzyme; TAPI-0, *N*-(*R*)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-naphthylalanyl-L-alanine amide.

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In the present study we have analysed more closely the role of proteolytic processing for L1 signalling. We observed in OVMz cells that ADAM10-mediated cleavage of L1 proceeds in both lipid rafts and in non-raft domains. In addition, we provide evidence that PS/γ-secretase activity is involved in further processing of the metalloprotease cleavage fragment. Pharmacological inhibition of ADAMs or γ-secretase activity blocked nuclear translocation of L1 and abrogated L1-dependent gene regulation. Moreover, specific targeting of ADAM10 and PS1 with siRNA (small interfering RNA) affected transcription of L1-dependent genes. We also demonstrate that overexpression of recombinant L1-ICD mediates gene regulation similarly to full-length L1. Our results establish, to our knowledge for the first time, that proteolytic processing by ADAMs and PS/γ-secretase is essential for nuclear signalling of L1 in cancer cell lines.

EXPERIMENTAL

Cells and DNAs

The ovarian carcinoma cell line OVMz and the stably transfected cell lines HEK-293-hL1 (human embryonic kidney-293-human L1) and CHO-hL1 (Chinese-hamster ovary-hL1) have been described previously [6,20]. Human pancreatic adenocarcinoma cells PT45-PI were described in [21]. Plasmids encoding PS1 and the dominant negative mutant (D385N) were obtained from Professor Dr Christian Haass (Laboratory for Alzheimer's and Parkinson's Disease, Department of Biochemistry, Adolf Butenandt Institute, Ludwig Maximilians University, Munich, Germany). A fragment encoding L1cyt (the cytoplasmic part of L1) from position Gly^{1148} to the C-terminus was constructed by PCR and both L1cyt and full-length L1 were inserted into the retroviral vector pBMIres-Puro. The transduction of cell lines with retroviral vectors and selection with puromycin was as described previously [22]. All cell lines were cultivated in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) FCS (fetal-calf serum) at 37 °C, 5 % CO_2 and 100 % humidity.

Chemicals and antibodies

Antibodies to the ectodomain (L1-11A) or cytoplasmic domain (pcytL1) of human L1 have been described [6,9]. The mAb (monoclonal antibody) 74 5H7 [23] to the cytoplasmic part of L1 was kindly provided by Professor Vance P. Lemmon (Miami Project to Cure Paralysis and Neuroscience Program, University of Miami Miller School of Medicine, Miami, FL, U.S.A.). The mAbs to the ectodomains of ADAM10 (MAB1427) and TACE (tumour-necrosis factor-α converting enzyme) (MAB9301) were from R&D (Wiesbaden, Germany), C-terminal pAbs (polyclonal antibodies) were from Chemicon International (AB19026 and AB19027 respectively). mAb 11G2 to ADAM10 was from Abcam (Cambridge, U.K.). The antibodies to nucleoporin, BIP/GRP78 (immunoglobulin heavy chain binding protein/78 kDa glucose regulatory protein), the small GTPase rab11, ERK and phospho-ERK were purchased from BD-Transduction (Heidelberg, Germany). The antibody to LAMP-1 (lysosomal-associated membrane protein 1) was from Santa Cruz (Heidelberg, Germany). The pAb to the C-terminus of PS (P7854) was from Sigma (Taufkirchen, Germany). Secondary antibodies were obtained from Dianova (Hamburg, Germany).

Biochemical analysis

The analysis of L1 cleavage was carried out as described previously [24]. Briefly, supernatants of a confluent cell monolayer were collected and subjected to precipitation with trichloroacetic acid. Cells were removed from the tissue-culture plastic surface with PBS/5 mM EDTA. Cell pellets were lysed in lysis buffer (20 mM Tris/HCl, pH 8.0, containing 1 % β-octyl glycopyranoside, 150 mM NaCl and 1 mM PMSF), cleared by centrifugation and mixed with twice-concentrated reducing SDS-sample buffer. SDS/PAGE under reducing conditions and transfer of separated proteins to Immobilon membranes using semi-dry blotting was as described previously [24]. After blocking with a 5 % (w/v) solution of dried skimmed-milk powder in TBS (Tris-buffered saline; 10 mM Tris/HCl and 150 mM NaCl, pH 8.0), the blots were incubated with the respective primary antibody followed by HRP (horseradish peroxidase)-conjugated secondary antibody and enhanced chemiluminescence detection.

Isolation and analysis of membrane lipid rafts

Cells (approx. 1×10^7) were lysed in 10 mM Tris/HCl, pH 8.0, containing 1 % Triton-X100, 150 mM NaCl, 1 mM PMSF and 1 μg/ml aprotinin at 4 °C. The lysate was mixed with an equal volume of sucrose solution (85 %, w/v, in TBS containing Boehringer Complete™) and 0.5 ml was transferred to a centrifuge tube. A step gradient was prepared by overlaying with 3 ml of 35 % sucrose in TBS, followed by 0.5 ml of 5 % sucrose in TBS as described in [25]. The gradient was centrifuged for 18 h at 200 000 ***g*** in a Beckman SW60 rotor. Fractions (0.5 ml each) were collected from top of the gradient and precipitated with chloroform/methanol as described in [25]. Samples were dissolved in SDS-sample buffer. SDS/PAGE, Western blotting and enhanced chemiluminescence were performed as described in [24].

siRNA transfection

siRNAs targeting L1, ADAM10 and TACE were described in [11,26]. siRNA specific for PS1 (AAGGUCCACUUCGUAUG-CUGGTT) was purchased from Eurofins MWG GmbH (Ebersberg, Germany). OVMz cells were transfected with Oligofectamine™ (Invitrogen) according to the manufacturer's protocol.

Quantitative real-time PCR

RNA was isolated using the RNAeasy Mini Kit (Qiagen, Hilden, Germany). For qRT-PCR (quantitative real-time PCR) the cDNA was purified on Microspin G-50 columns [Amersham Biosciences (now GE Healthcare), Freiburg, Germany] and quantified using a NanoDrop spectrophotometer (ND-1000; Kisker-Biotechnology, Steinfurt, Germany). Primers for qRT-PCR were designed with the DNA Star Program and were produced by Eurofins MWG. β-actin was used as an internal standard. The PCR reaction was performed with the SYBR Green Master Mix (Applied Biosystems, Darmstadt, Germany). The sequences of the primers used are available from P.A. on request.

Flow cytometry and immunofluorescence microscopy

The staining of cells with mAbs and phycoerythrin-conjugated secondary antibodies has been described [6]. Cells were analysed with a FACScan flow cytometer using Cellquest software (BD Biosciences, Heidelberg, Germany). For confocal microscopy, cells were fixed with 3 % (v/v) paraformaldehyde/PBS for 20 min, quenched for 10 min in 50 mM NH_4Cl, permeabilized for 5 min with methanol (−20 °C) and stained with pcytL1 and Alexa488-conjugated anti-rabbit IgG. Cell preparations were mounted in Mowiol and images were acquired using a confocal laser imaging system (LSM 510; Carl Zeiss MicroImaging). Quantification was performed using ImageJ software. Nuclei were bordered in phase contrast and the pixel number within the encircled area was determined.

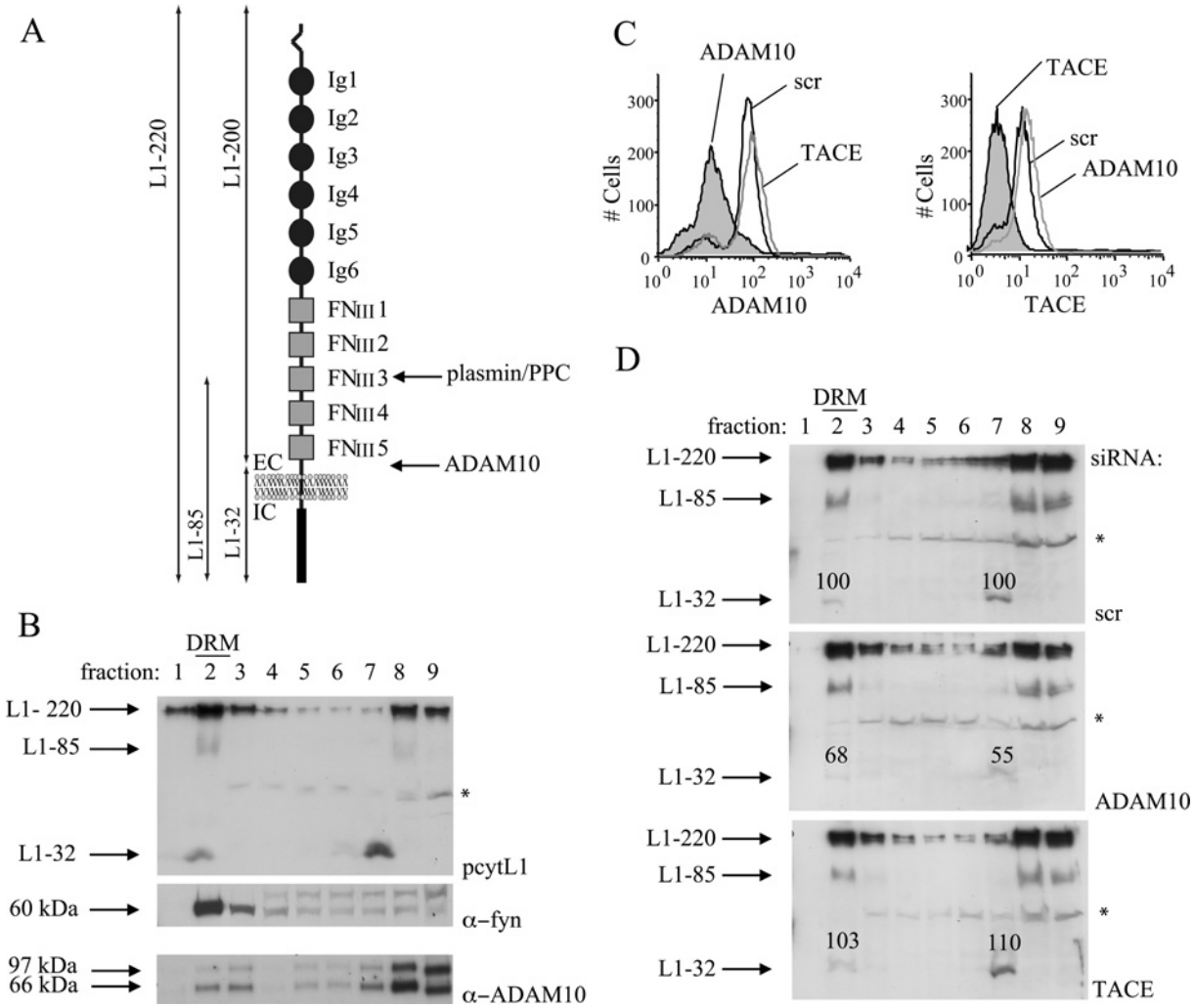

Figure 1 L1 cleavage in OVMz cells is mediated by ADAM10

(**A**) Schematic illustration of L1 cleavage fragments. Abbreviations: Ig, immunoglobulin; PPC, proprotein convertase. (**B**) OVMz cells were lysed in 1 % Triton X-100. The sample was mixed with an equal volume of 85 % sucrose, placed on the bottom of a centrifuge tube and overlaid with a stepwise sucrose gradient as described in the Experimental section. After ultracentrifugation, fractions were harvested from the top of the gradient and analysed by SDS/PAGE and Western blotting. Fractions were probed with pcytL1 to the cytoplasmic portion of L1, followed by HRP-conjugated secondary antibodies and ECL® detection. The nomenclature of L1 cleavage fragments is as described previously [6]. Gradient fractions were re-probed with antibodies to fyn and ADAM10. The mature form (66 kDa) and the pro-form (97 kDa) are indicated. (**C**) OVMz cells were transfected with siRNA specific for ADAM10 or ADAM17. Expression levels were analysed 48 h after transfection by flow cytometry with mAbs to the ectodomains of ADAM10 or ADAM17. Abbreviation: scr, scrambled control siRNA. (**D**) siRNA-transfected cells were lysed in Triton X-100 and sucrose-density-gradient centrifugation was performed as described above. Gradient fractions were analysed by Western blot with pcytL1 as described above. Numbers indicate relative band intensities. The asterisk (*) indicates non-specific bands.

Purification of nuclei

Purification of nuclei was performed as described in [27]. Nuclear extracts were prepared by incubation of the purified nuclei in high-salt buffer [0.42 M NaCl/20 mM Hepes (pH 7.9)/ 25 % (v/v/) glycerol] for 30 min at 4 °C, followed by centrifugation (10 000 ***g*** for 30 min at 4 °C).

Statistical analysis

For the analysis of statistical significance, the Student's *t* test was used.

RESULTS

Cleavage of L1 in lipid raft and non-raft domains

L1 is expressed as a full-length molecule (L1-220), but can be proteolytically cleaved at different sites as outlined in Figure 1(A). Cleavage in the third FNIII (fibronectin III) domain generates a fragment of 85 kDa (L1-85). In addition, a 32 kDa (L1-32) fragment results from membrane proximal cleavage mediated by ADAM10. We initially investigated whether L1-220 was associated with lipid rafts using solubilization in Triton X-100 and sucrose-density-gradient centrifugation. In OVMz cells a significant portion of full-length L1-220 (between 20 and 30 %, $n > 10$) was recovered in the low-density DRM (detergent-resistant membrane) fraction, identified by the marker tyrosine-specific phosphotransferase fyn (Figure 1B).

The L1-32 cleavage fragment was detected in both lipid raft (fraction no. 2) and non-raft fractions floating above the loading zone (fraction no. 7). A similar distribution of L1-220 and L1-32 was found in other cell lines such as the breast carcinoma cell line AR [24], stably transduced CHO-hL1, HEK-293-hL1 and pancreatic-carcinoma PT45-PI-hL1 cells (results not shown).

Cleavage of L1 by ADAM10 in lipid rafts and non-rafts

The above results indicated that L1 distributes between lipid raft and non-raft domains and that membrane proximal cleavage is apparent in both. We next analysed the distribution of ADAM10, the protease responsible for constitutive shedding of L1. A subpopulation of mature ADAM10 (66 kDa) was associated with lipid rafts and co-localized with L1-32 (Figure 1B). In addition, ADAM10 was detected in the bottom fraction of the gradient. To test whether ADAM10-mediated processing is restricted to lipid rafts, we targeted ADAM10 with a specific siRNA. As a control, cells were treated with siRNA to ADAM17, which catalyses the induced shedding of L1 [11]. Efficient knock-down was confirmed by flow cytometry (Figure 1C) and Western-blot analysis (results not shown). Down-regulation of ADAM10, but not ADAM17, decreased the level of L1-32 in both lipid raft and non-raft fractions (Figure 1D). These results suggested that constitutive shedding of L1 in OVMz cells is independent of lipid-raft localization.

L1-32 co-fractionates with late endosomal marker LAMP-1

We noticed that fraction no. 7 of the raft gradient contained L1-32, whereas L1-220 was hardly detectable (Figure 1B). To further define the composition of fraction no. 7, we used an established fractionation protocol to separate early and late endosomes and heavy membranes (Figure 2A) [28]. L1-32 was enriched at the 8/25%-sucrose interface, where it co-fractionated with the late endosomal marker LAMP-1 (Figure 2B). The marker protein EEA-1 (early endosomal antigen 1) was not detected, suggesting that fraction no. 7 was devoid of early endosomes. By contrast, rab11, a marker for recycling endosomes, was detected at the 25/35 % interface. Co-fractionation of LAMP-1 and L1-32 suggested that late endosomes/lysosomes are candidate sites for the proteolysis of L1.

L1-32 is a substrate for PS/γ-secretase activity

Previous work in fibroblastic mouse cell lines had shown that L1-32 undergoes further processing by PS/γ-secretase [12]. The active γ-secretase complex is associated with lipid rafts [29]. The PS1 holoprotein (48 kDa) is endoproteolysed to generate N-terminal (25–25 kDa) and C-terminal (18 kDa) fragments. Both fragments are found in high-molecular-mass complexes with other integral membrane proteins, which together constitute the active γ-secretase.

When we analysed the distribution of PS1 in sucrose-gradient fractions, the 48 kDa holoprotein was detected in lipid rafts, whereas the 18 kDa C-terminal fragment was mainly found in the bottom fractions (Figure 3A). We used the specific PS inhibitor IX (DAPT {*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine t-butyl ester}) to investigate cleavage of L1 in OVMz cells. The treatment led to an increase in L1-32 (Figure 3B). This was not due to enhanced ADAM10 cleavage, but rather the effect of blocking L1-32 processing, since the amount of soluble L1 in the medium was not increased (Figure 3C). In agreement with this notion, the accumulation of L1-32 by DAPT was also apparent when metalloprotease cleavage was blocked by the inhibitor TAPI-0 {*N*-(*R*)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-naphthylalanyl-L-alanine amide}, ruling out the possibility that DAPT itself activated metalloproteinase cleavage (results not shown). Overexpression of PS1 led to a significant decrease in L1-32 in both lipid raft and non-raft domains. By contrast, dominant-negative PS1 (D385N) caused the accumulation of L1-32 (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/420/bj4200391add.htm), an observation that supports a role for PS1 in L1-processing.

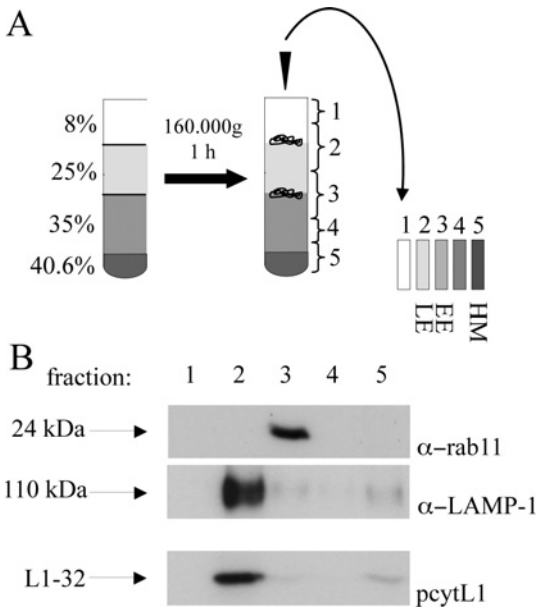

Figure 2 L1-32 co-fractionates with the late endosomal marker LAMP-1

(**A**) Fraction no. 7 of the raft gradient was harvested, diluted with PBS and subjected to further ultracentrifugation for 2 h at 100 000 ***g***. The pellet was suspended in 0.5 ml of 40.6 % sucrose and loaded into the bottom of an SW60 centrifuge tube. This was overlaid with 1.5 ml of 35 % sucrose, followed by 1 ml of 25 % sucrose and, finally, 0.5 ml of 8 % sucrose. The gradient was centrifuged for 1 h at 160 000 ***g*** in a Beckman SW60 rotor. Fractions (1 ml each) were collected from the top of the gradient and precipitated with chloroform/methanol. Late endosomes (LE) were enriched at the 8/25 %-sucrose interface, early endosomes (EE) at the 25/35 % interface and heavy membranes (HM) between 35 and 40.6 %. (**B**) Gradient fractions were probed with pcytL1 and antibodies (α-) to LAMP-1 and rab11.

It was reported previously that PS/γ-secretase cleavage of L1-32 released a smaller C-terminal fragment of 28 kDa in mouse fibroblasts [12]. In lysates of OVMz or HEK-293-hL1 cells, L1-28 was difficult to detect, most probably because of rapid degradation and/or low abundance. Previous studies have indicated that the ICDs of γ-secretase-cleaved proteins are rapidly degraded by cytosolic proteases [30,31].

To directly prove that L1-32 is a substrate for γ-secretase, we analysed the processing of L1-32 in microsomal membranes to exclude degradation by cytosolic proteases. CHO-hL1 cells were cultivated for 16 h in the presence of DAPT or DMSO and membranes were isolated and assayed for *in vitro* γ-secretase activity [32]. Isolated membranes were incubated for 2 h at 37 °C and the membranous and soluble fractions were separated by ultracentrifugation. We identified L1-28 in the supernatant fraction (Figure 3D, lane 2) of membranes isolated from DMSO-treated cells. By contrast, no such fragment was detected in membranes prepared from cells incubated with the PS inhibitor DAPT. Instead, L1-32 was found in the membrane pellet (Figure 3D, lane 3). Similar observations were made in OVMz cells (results not shown). These findings suggested that L1-32 is further cleaved by γ-secretase, resulting in the release of L1-28, representing the L1-ICD without the membrane portion.

Nuclear accumulation of L1-ICD

Consecutive cleavage by both enzymes is a hallmark of Notch, APP and CD44 signalling, which is followed by translocation of the ICD to the nucleus [13]. As L1 affects gene expression,

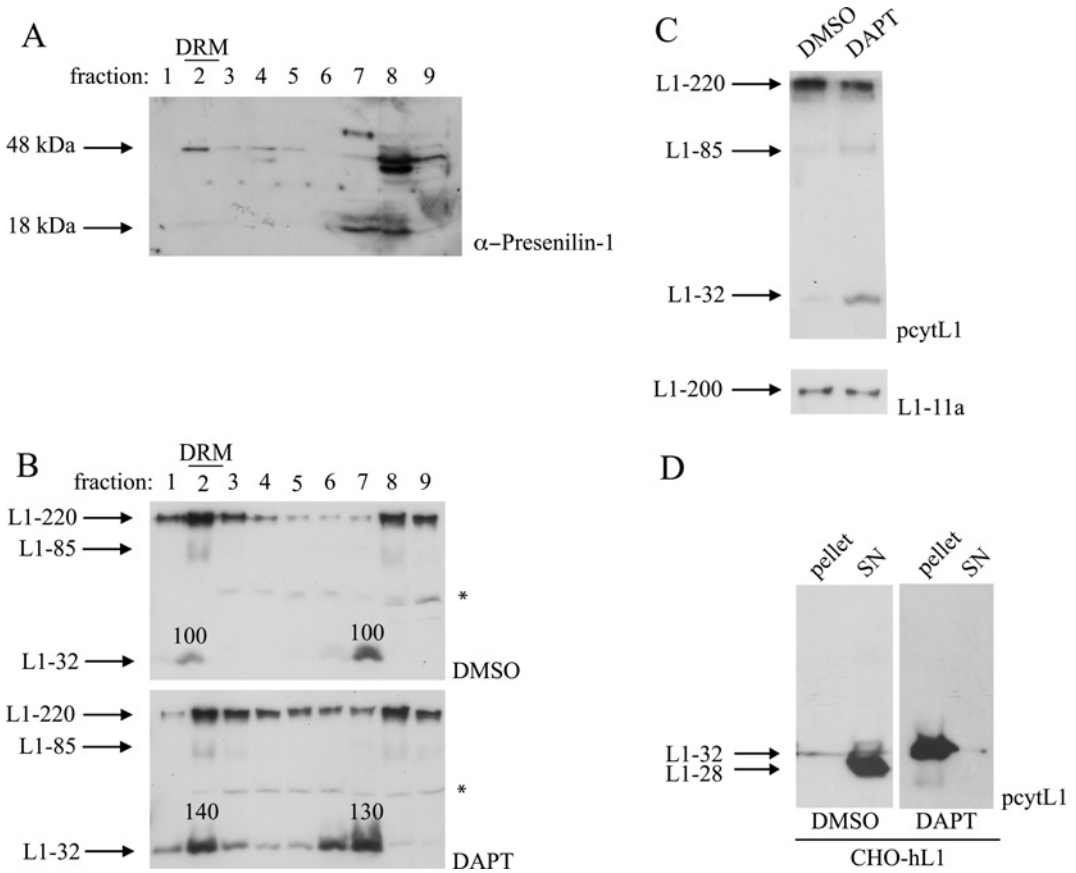

Figure 3 PS/γ-secretase inhibitors block L1-32 processing

(**A**) OVMz cells were subjected to sucrose-density-gradient centrifugation as described in Figure 1 and the distribution of PS1 was determined. (**B**) OVMz cells were incubated for 24 h with the PS inhibitor DAPT or the solvent DMSO. Cell lysates were prepared in lysis buffer containing Triton X-100 and sucrose-density-gradient centrifugation was carried out as described above. Gradient fractions were analysed by Western blotting with pcytL1, followed by HRP-conjugated secondary antibodies and ECL® detection. (**C**) OVMz cells were treated for 24 h with PS inhibitor IX/DAPT or the solvent DMSO and lysates were analysed with pcytL1. Soluble L1 was precipitated from the tissue-culture medium and detected by mAb L1-11A to the ectodomain of L1, followed by HRP-conjugated secondary antibodies and ECL® detection. (**D**) Cells were grown for 48 h at 37 °C in the presence of DAPT or DMSO. Isolated membranes were incubated for 2 h at 37 °C and separated into pellet or supernatant (SN) fractions by ultracentrifugation. Lanes 1 and 2 show membranes isolated from DMSO-treated cells (vehicle); lanes 3 and 4 show membrane preparations from cells incubated with DAPT. Numbers indicate relative band intensities. The asterisk (*) indicates non-specific bands.

we investigated the nuclear localization of L1-ICD. Nuclei were purified using a well-established protocol [27]. The purity of the nuclear fractions was examined by Western blotting using marker proteins. A representative purification is shown in Figure 4(A). We observed that isolated nuclei were not contaminated by cytosolic proteins (marker moesin) or endoplasmic-reticulum-specific proteins (BiP/GRP78 marker). Nucleoporin (a nuclear marker) was exclusively present in the nuclear fraction. Using pcytL1 antibody to the cytoplasmic portion of L1, we detected L1-ICD in the nuclear fraction of OVMz (Figure 4B). When isolated nuclei were extracted with high salt and separated into soluble and insoluble fractions by centrifugation, L1-ICD was found exclusively in the soluble fraction. The L1-ICD fragment was also detected in HEK-293-hL1 but was absent in L1-negative HEK-293 cells (Figure 4C).

Nuclear translocation of L1 is blocked by inhibitors of metalloproteases and PS/γ-secretase

We performed confocal microscopy to verify the nuclear localization of L1. Using pcytL1 to the cytoplasmic portion of L1, staining was detected in the cytoplasm and the nucleus of CHO-hL1 and OVMz cells that was absent in CHO control cells (Figure 4D). Next, we studied whether the inhibition of metalloprotease and/or PS activity could block L1 nuclear translocation. Treatment with the single compounds or in combination caused a decrease of L1 staining in the nucleus (Figure 5A). Quantification by image analysis revealed a time-dependent decrease of L1 nuclear staining that was detectable after 48 h of treatment and was even more pronounced after 72 h (Figures 5B, 5C and 5D).

Metalloprotease and PS cleavage are essential for L1-mediated gene regulation

We investigated whether inhibition of L1-processing affects L1-dependent transcriptional regulation. We have recently identified a set of transcripts in HEK-293 cells, including CRABPII (cellular retinoic acid-binding protein II), β3 integrin and cathepsin B, which are regulated by expression of L1 [9]. HEK-293 and HEK-293-hL1 cells were grown for 96 h in the presence of DAPT, TAPI-0 or both inhibitors. qRT-PCR analysis showed that L1-regulated transcription of cathepsin B and CRABPII was blocked by TAPI-0 or DAPT, or both (Figure 6A).

As ERK activation appears to be required for L1-mediated transcriptional regulation, we analysed the phosphorylation of ERK

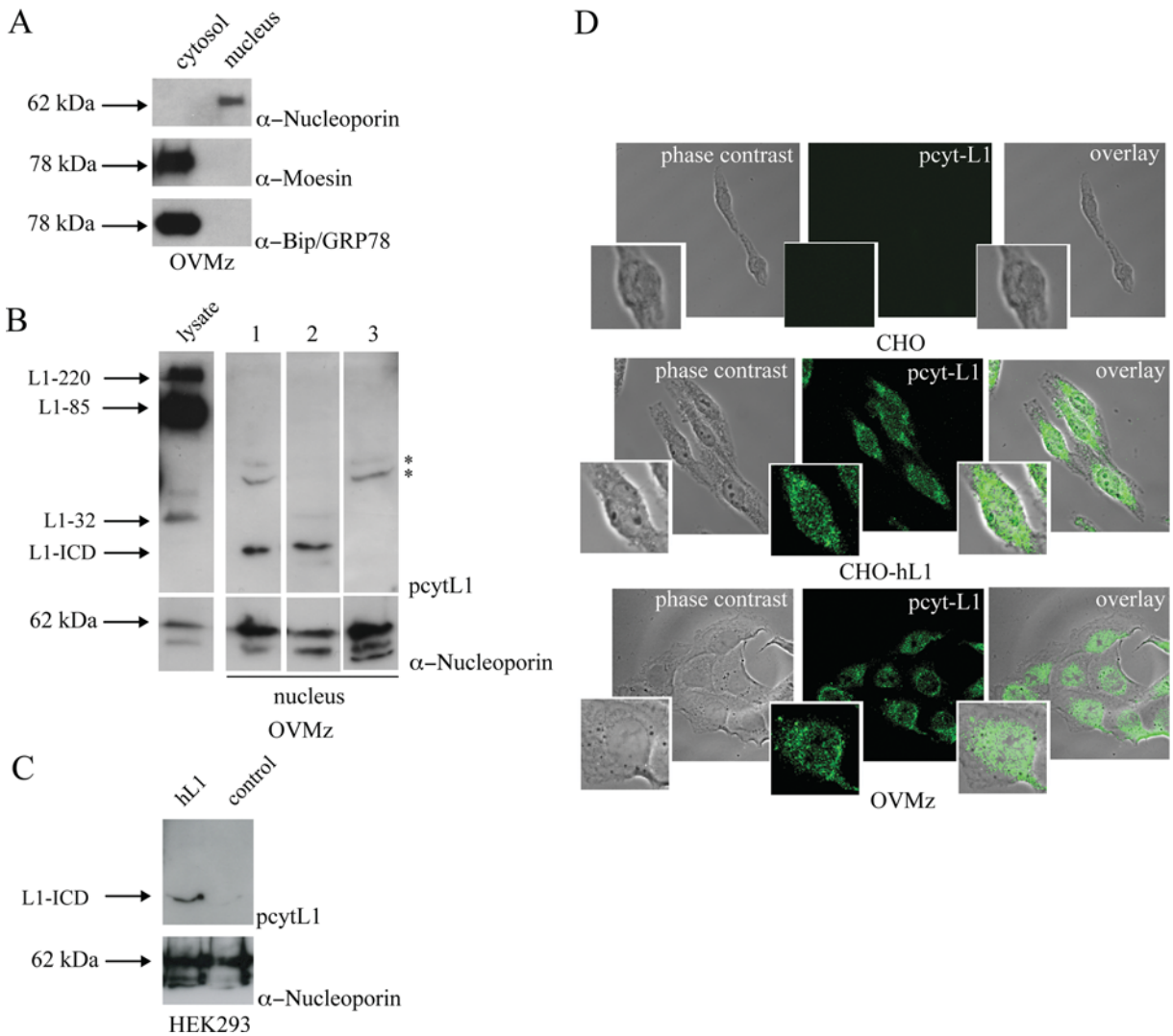

Figure 4 Nuclear translocation of L1-ICD

(**A**) Purity of isolated nuclei as revealed by marker-protein analysis. (**B**) L1-ICD in the nucleus. OVMz cells were grown in 10 % FCS, and nuclei were prepared and analysed by Western blotting with pcytL1 (lane 1). Isolated nuclei were extracted with 0.42 M NaCl, and soluble proteins (lane 2) and insoluble membranes (lane 3) were separated by centrifugation. (**C**) HEK-293 and HEK-293-hL1 cells were cultivated in 10 % FCS, and nuclei were isolated and analysed by Western blotting with pcytL1. (**D**) Nuclear localization of L1 in CHO-hL1 cells (middle row) and OVMz cells (bottom row). L1-negative CHO cells were used as control (top row). Cells were fixed with 3 % paraformaldehyde, permeabilized with methanol (−20 °C) and stained with pcytL1 and Alexa488-conjugated anti-rabbit IgG. Note that methanol treatment eliminates membranous L1 staining, but leaves intact intracellular and nuclear staining. The asterisk (*) indicates non-specific bands.

in treated cells. Importantly, neither compounds affected ERK activation in HEK-293 or HEK-293-hL1 cells (Figure 6B). These results clearly showed that the changes in L1-mediated gene regulation were independent of ERK.

Cleavage of L1 is essential for L1-mediated gene regulation in carcinoma cells

To extend the above findings on ovarian carcinoma cells, we transiently down-regulated L1 in OVMz cells (Figure 7A) and screened for genes that were differentially regulated compared with mock-transfected cells. DNA-array analysis identified 282 genes out of 46 713 that were up- or down-regulated (S. Wolterink, unpublished work). L1-dependent expression of a set of genes was confirmed by qRT-PCR. Figure 7(B) shows altered expression levels of transcripts for MDK (midkine or neurite growth-promoting factor 2) and β3 integrin after knock-down of L1 by specific siRNA.

To corroborate that consecutive cleavage of L1 is essential for L1-mediated gene regulation, OVMz cells were transfected with siRNAs targeting ADAM10 or PS1. Efficient down-regulation of ADAM10 and PS1 was confirmed by Western blotting (Figure 7C). Targeting of PS1 caused an accumulation of L1-32 (results not shown), an observation in agreement with what has been observed after overexpression of dominant-negative PS1 (D385N) (see Supplementary Figure S1) or treatment with DAPT (see Figure 3B). Down-regulation of ADAM10 or PS1 affected transcription of MDK and β3 integrin similarly to down-regulation of L1 (Figure 7D).

Recombinant L1-ICD mediates transcriptional regulation

On the basis of these findings we hypothesized that an artifical L1-ICD might be able to promote L1-dependent transcriptional regulation. To test this, we constructed L1cyt, a cytoplasmic fragment of L1 lacking the ectodomain and transmembrane

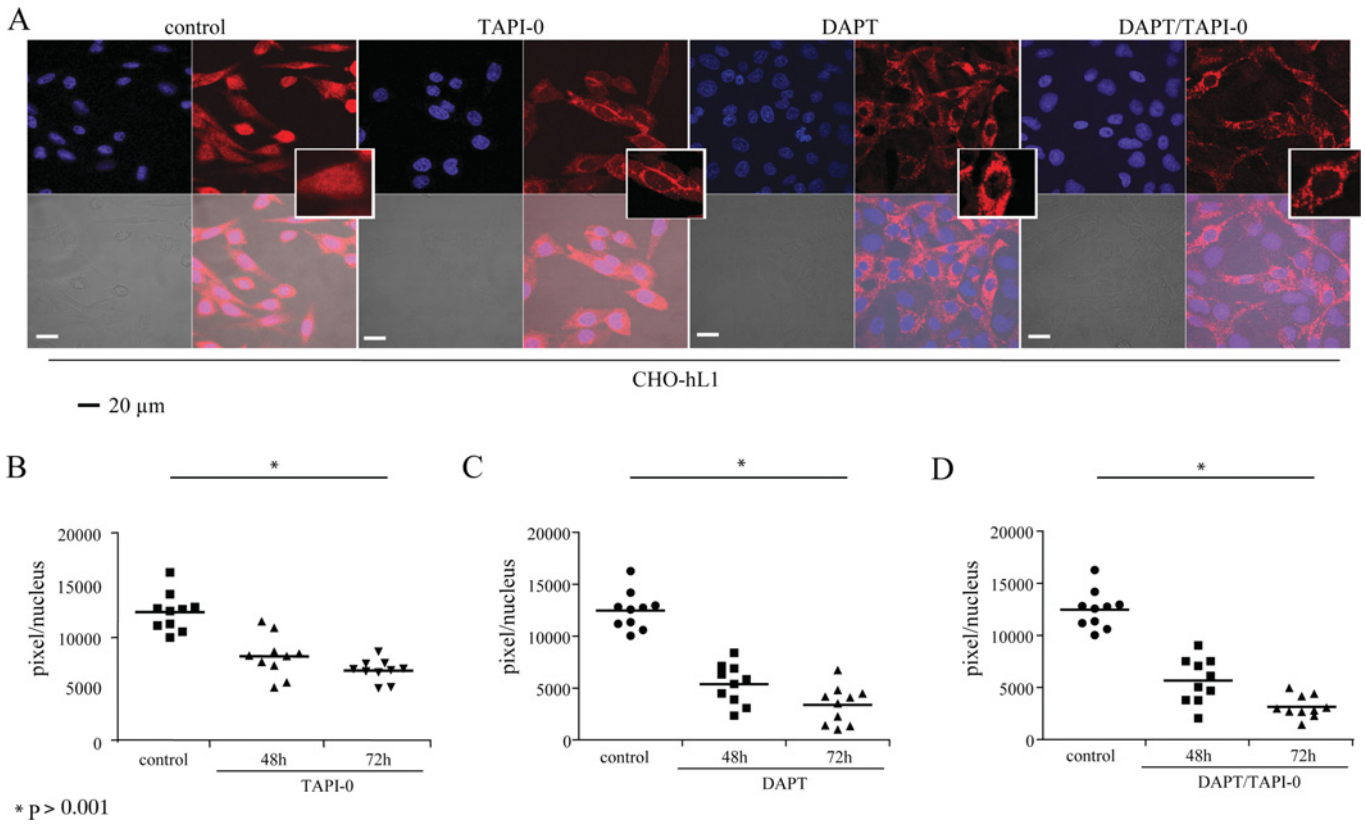

Figure 5 Inhibitors of metalloproteases and PS block L1 nuclear translocation

(**A**) CHO-hL1 cells were treated for 72 h with TAPI-0, DAPT or TAPI-0/DAPT and nuclear localization of L1 was analysed. Cells were fixed with 3 % paraformaldehyde, permeabilized with methanol (−20 °C), stained with pcytL1 and Alexa488-conjugated anti-rabbit IgG and analysed by confocal microscopy. Note that methanol treatment abolishes membranous L1 staining, but leaves intact intracellular and nuclear staining. (**B**–**D**) Quantification of nuclear staining of L1 in CHO-hL1 cells treated with the indicated compounds for 48 or 72 h. For each analysis, ten nuclei were encircled and the pixel number in the nuclei was determined. CHO-hL1 cells were used because they were easier to handle in microscopic analysis.

region. Cells were stably transduced with L1cyt and full-length L1. Intracellular staining using mAb 745H7 to the cytoplasmic portion of L1 monitored by flow cytometry showed that both constructs were expressed at comparable levels (Figure 8A). Western blot analysis demonstrated that the L1cyt fragment had a size of approx. 30 kDa and migrated slightly smaller than the L1-32 fragment generated by proteolytic cleavage of full-length L1 (Figure 8B). Biochemically, L1cyt was detectable in purified nuclei and was very similar in size to the L1-ICD fragment released after γ-secretase mediated cleavage of L1-32 (Figure 8C). Importantly, L1cyt affected gene expression of CRABPII and β3 integrin similarly to full-length L1, as shown by qRT-PCR. Neither L1cyt nor full-length L1 altered cathepsin B gene expression in PT45-PI cells (Figure 8D).

DISCUSSION

The cell-adhesion molecule L1 is a type 1 transmembrane protein that is expressed in human carcinomas and melanomas and has been linked to poor prognosis [33,34]. L1 undergoes regulated proteolysis at the cell surface and in released exosomes, a process that involves the metalloprotease ADAM10 [6,10,11]. Both L1 and ADAM10 were detected at the invasive front of human colon-cancer tissue [35], and transfection of ADAM10 confers metastatic capabilities on colon cancer cells expressing L1 [36]. In addition, ADAM10 is expressed in ovarian and uterine carcinomas in which soluble L1 is detectable in the serum [34].

Little is known about the fate of the L1-32 that remains after membrane proximal cleavage. Numerous studies have shown that residual transmembrane remnants become susceptible to RIP. Many substrates for PS-dependent γ-secretase have already been identified, such as APP [37], Notch [30], ErbB4 (v-erb-a erythroblastic leukaemia viral oncogene homologue) [38,39], E-cadherin [40] and CD44 [41]. They all undergo ectodomain shedding, which is evidently a prerequisite for subsequent γ-secretase cleavage. Likewise, recent studies in mouse fibroblastic cell lines demonstrated that L1 is further processed by the PS–γ-secretase complex after initial cleavage by ADAM10 [12].

Here we provide evidence that regulated intramembrane proteolysis of L1 is also apparent in ovarian carcinoma cells. The decrease in PS/γ-secretase activity brought about by the specific inhibitor DAPT or the overexpression of dominant-negative PS1 (D385N) led to the accumulation of L1-32 without a concomitant increase in the L1 ectodomain. Moreover, we observed in an *in vitro* assay that the conversion of the initial ADAM10 cleavage fragment L1-32 into a soluble form of approx. 28 kDa is sensitive to the γ-secretase inhibitor DAPT. In contrast with the findings by Maretzky et al. [12], we were not able to detect L1-28 in cell lysates, even when cells were grown in the presence of proteasome inhibitors (results not shown). This might be due to low abundance and/or rapid degradation of the L1 intracellular domain in carcinoma cells. However we identified an L1 cleavage product after enrichment of purified nuclei. The size of this fragment was similar to that of the soluble L1-form released in the *in vitro* assay described.

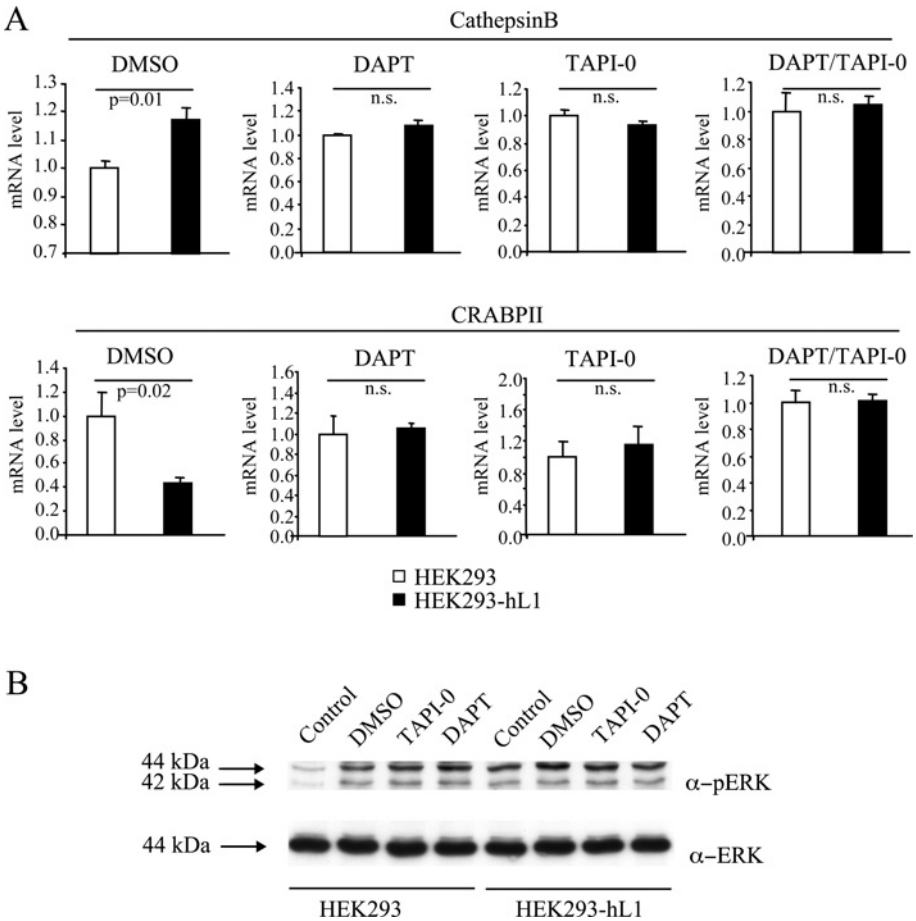

Figure 6 Inhibitors of metalloproteases and PS block L1-dependent gene regulation

(**A**) HEK-293 or HEK-293-hL1 cells were treated with DMSO, DAPT, TAPI-0, or both DAPT and TAPI-0, for 96 h. RNA was transcribed to cDNA and expression of CRABPII and cathepsin B was analysed by qRT-PCR. (**B**) Analysis of ERK phosphorylation in HEK-293 and HEK-293-hL1 cells treated with the indicated compounds.

We further analysed the role of proteolytic processing in L1-dependent signalling. Confocal microscopy confirmed that L1-ICD can localize to the nuclei of CHO-hL1 cells. Nuclear translocation was clearly diminished in cells treated with TAPI-0 and DAPT, inhibitors of metalloproteases and γ-secretase respectively. These findings suggested that L1 enters the nucleus after initial ADAM10 cleavage, followed by RIP. We assumed that L1-ICD in the nucleus might participate in transcriptional regulation by analogy to what has already been described for Notch [42]. To address this question, we investigated the impact of blocking proteolytic processing on L1-dependent gene regulation. We have recently identified a number of genes that are regulated by L1 in HEK-293 cells, such as those coding for cathepsin B, β3 integrin, CRABPII or HOXA9 (homeobox A9) [9]. As demonstrated here for cathepsin B and CRABPII, L1-mediated gene regulation was blocked in the presence of specific ADAM and PS/γ-secretase inhibitors. Moreover, specific targeting of ADAM10 or PS1 with siRNA affected the transcription of genes, e.g. those encoding MDK and β3 integrin, similar to knock-down of L1 in ovarian carcinoma cells. Taken together, these results highlight the relevance of consecutive processing for the downstream signalling of L1.

To corroborate the role of the L1-ICD in transcriptional regulation, we expressed an artificial L1-ICD lacking the ectodomain and transmembrane region (L1cyt). Indeed, we found that L1cyt localized to the nucleus and mediated transcriptional regulation similarly to full-length L1. Additional analysis has shown that L1cyt does not support cell proliferation *in vitro* and tumour growth in mice as does full-length L1 (H. Kiefel, unpublished work). These results are in agreement with recent observations by Gavert et al. [36]. Nevertheless, our findings underline the role of the cytoplasmic part of L1 for signalling and establish a link between proteolysis, nuclear translocation and L1-mediated transcriptional regulation. It is currently not clear how L1 signal transduction by RIP relates to the L1-dependent ERK signalling pathway previously characterized [7,9,43]. ERK1/2 are serine-threonine kinases that phosphorylate many cellular proteins, i.e. transcription factors, cytoskeletal proteins, membrane proteins and other kinases [44,45]. Activated ERK translocates from the cytoplasm to the nucleus, where it regulates gene transcription [45]. Of note, in previous work we have characterized a cytoplasmic L1 mutant (hL1mutTS) devoid of the ERK phosphorylation site (Ser^{1248}) that does not support L1-dependent gene regulation and ERK activation [9]. We observed recently that L1mutTS-ICD does not translocate to the nucleus, although it is processed by ADAM10 [46]. In the present paper we found that DAPT or TAPI-0 treatment of L1-expressing HEK-293 cells did not affect ERK phosphorylation (Figure 6B). Similar observations were made when L1 was specifically depleted using siRNA [26]. Although these results might suggest a less important role in ERK activation, it is still possible that L1 and ERK have to co-operate for L1 processing and/or

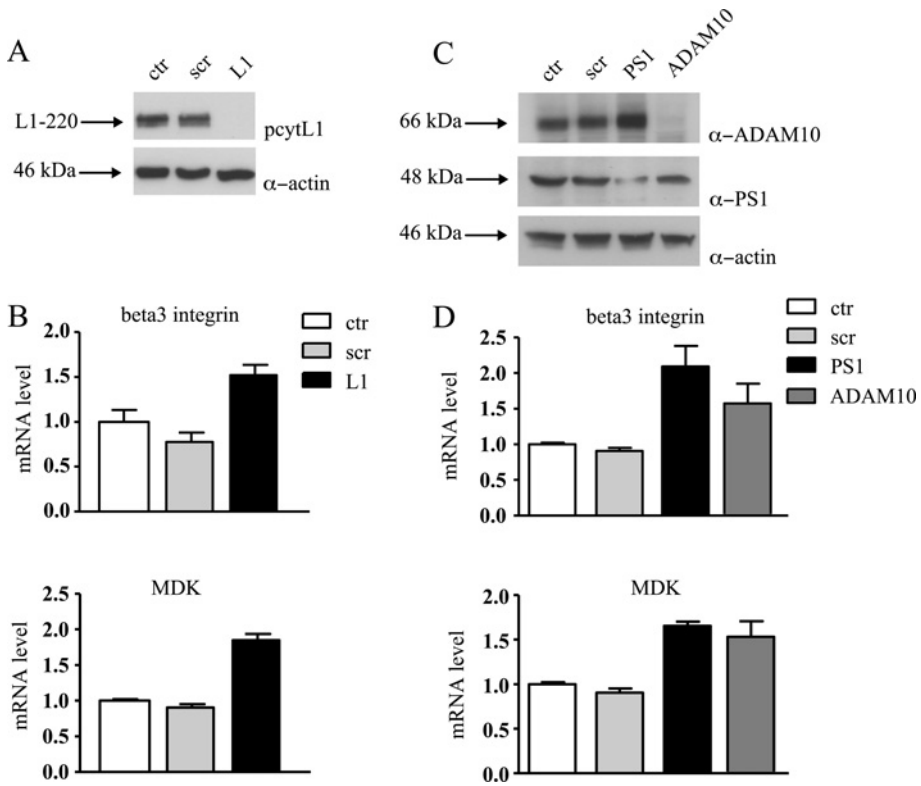

Figure 7 Targeting of PS1 or ADAM10 blocks L1-dependent gene regulation

(**A**) OVMz cells were transfected with siRNA targeting L1. The knock-down of L1 was analysed 72 h after transfection using pcytL1 and ECL® detection. (**B**) RNA was transcribed to cDNA and expression of MDK and β3 integrin was analysed by qRT-PCR. (**C**) OVMz cells were transfected with siRNA targeting PS1 or ADAM10. Knock-down was confirmed with antibodies to ADAM10 (mAb 11G2) or PS1. (**D**) RNA was transcribed to cDNA and expression of MDK and β3 integrin was analysed by qRT-PCR. One representative experiment is shown ($n = 3$).

transcriptional regulation. Further studies will need to uncover the contribution of each mechanism to the outcome of L1 signalling.

As lateral segregation of membrane proteins from their sheddases by cholesterol-rich microdomains seems to be a mechanism to control proteolysis [47], we investigated the importance of L1 partition into lipid rafts for its ectodomain shedding. Using sucrose-density-gradient centrifugation, we showed that L1 distributes between lipid raft and non-raft domains. Moreover, we detected the proteolytic fragment L1-32 in both lipid raft and non-raft fractions. Knock-down of ADAM10 diminished the cleavage fragment in both membrane domains, whereas down-regulation of TACE had no effect. These data indicated that L1 is cleaved by ADAM10 independently of its distribution into lipid rafts. A similar finding was described for MT1-MMP (membrane type-1 matrix metalloproteinase), a membrane-anchored protease that is proteolytically released from the cell surface. The latter process is independent from lipid rafts, since both transmembrane and glycosyl-phosphatidylinositol-anchored MT1-MMP are shed [48]. Although partition of L1 into lipid rafts seems not to be crucial for its ectodomain shedding, disruption of lipid raft integrity by the cholesterol-binding drug nystatin completely abrogated L1 processing in these microdomains (S. Riedle, unpublished work).

L1-32 was also detected in non-raft domains floating above the loading zone (Figure 1B). Refined subcellular separation demonstrated that L1-32 co-fractionated with LAMP-1, a marker for late endosomes/lysosomes. By contrast, full-length L1 was hardly detectable in this fraction. L1-32 may be delivered to late endosomes/lysosomes selectively. It is also conceivable that full-length L1 is completely cleaved within these compartments. Growing evidence suggests a role for endosomes in the proteolysis of membrane proteins [49]. Further analysis has to define the subcellular localization of L1-cleavage. Nevertheless, identification of L1-32 in late endosomes/lysosomes suggests that these endocytic compartments are likely candidate sites for the proteolytic processing of L1.

Interestingly, the active γ-secretase complex is also present in lipid rafts, although it seems not to be cholesterol-dependent [29]. We therefore investigated whether the PS/γ-secretase-dependent processing of L1 is associated with lipid rafts. Using sucrose-density-gradient centrifugation we were unable to detect any considerable amount of PS1 CTF (C-terminal fragment) in the lipid raft fraction. This might be caused by the detergent used for the preparation, since the choice of detergent is critical for the detection of PS1 in lipid rafts. Mature components of the γ-secretase complex are soluble in Triton X-100, but remain insoluble in Lubrol-WX [50]. Nevertheless, we observed an increase of L1-32 in the lipid raft fraction after DAPT treatment. Importantly, this accumulation was not restricted to lipid rafts, but was also apparent in non-raft domains. Overexpression of dominant-negative PS1 (D385N) confirmed these results. A similar result was found for embryonic mouse brains, where the majority of PS1, nicastrin (part of the secretase complex) and APP CTF remains soluble in Lubrol-WX [50]. Therefore our results support the notion that PS/γ-secretase cleavage is not restricted to proteins that are localized in lipid rafts.

The biological significance of the lateral segregation of L1 into lipid raft and non-raft domains is presently unclear, but this

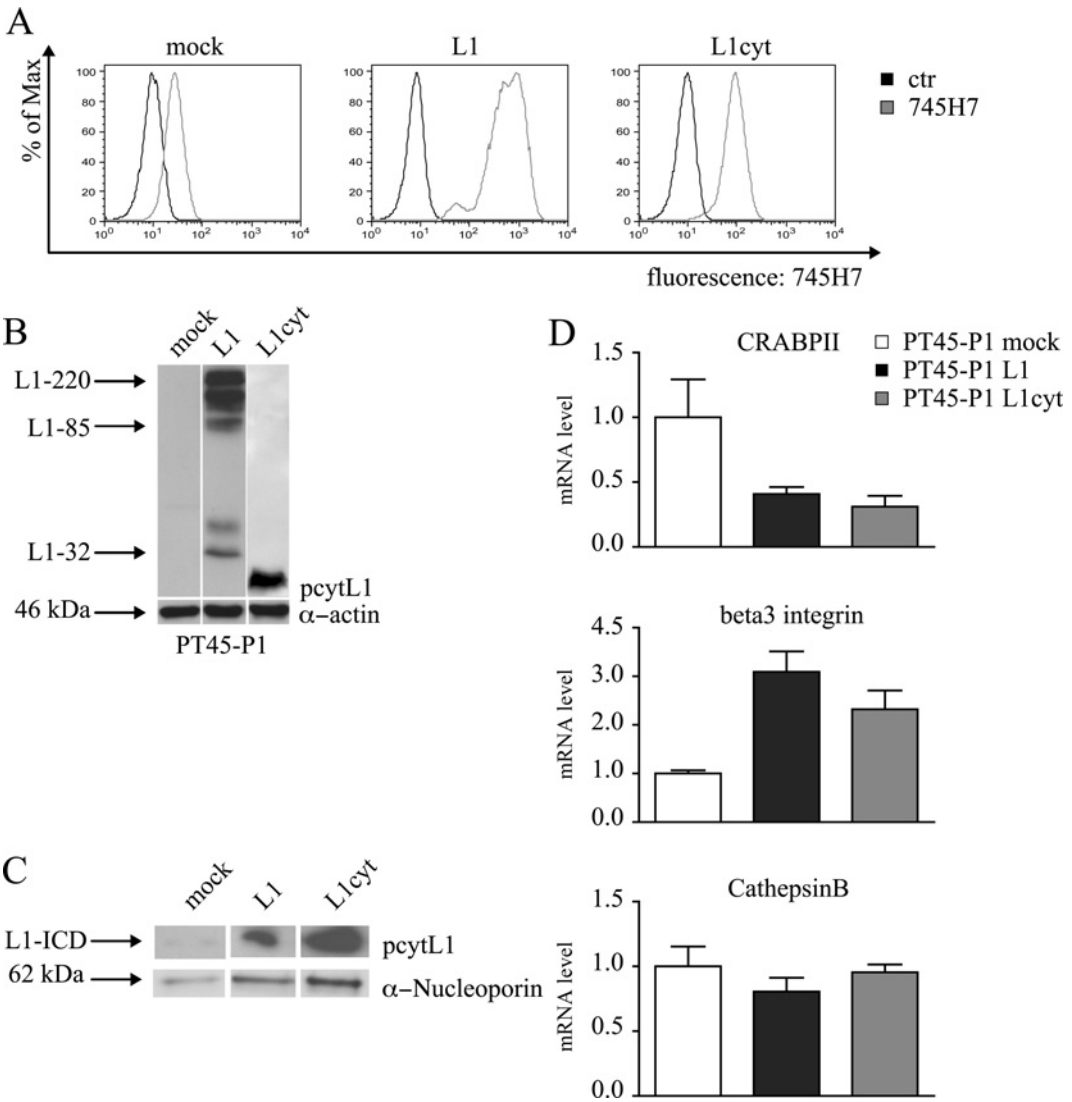

Figure 8 An L1 cytoplasmic fragment mediates transcriptional regulation

PT45-PI cells were transduced with retroviral vectors encoding L1cyt or full-length L1. (**A**) Expression levels were analysed by intracellular staining with a mAb (745H7) to L1, followed by flow cytometry. (**B**) Western blot analysis with pcytL1, followed by HRP-conjugated secondary antibodies and ECL® detection. (**C**) Detection of L1-ICD in isolated nuclei. Nuclei were prepared and immunoblotted with pcytL1 as described in the legend to Figure 4. (**D**) RNA was isolated and transcribed to cDNA. Expression of CRABPII, cathepsin B and β3 integrin was analysed by qRT-PCR. One representative experiment is shown ($n = 3$).

segregation has been reported in neuronal cells for L1 and the related molecule neurofascin [19,51]. Our preliminary results suggest that raft resident L1 associates with caveolin-1 (S. Riedle, unpublished work). It should be noted that endocytosis of L1 via the clathrin-mediated pathway has been well characterized [52]. It is therefore tempting to speculate that distinct endocytic pathways are involved in the internalization of L1. Such different routes regulate the signalling and turnover of transforming-growth-factor-β receptor [53] and bone-morphogenic-protein receptor [54]. Further work needs to clarify whether the segregation of L1 into lipid raft and non-raft regions is required for its internalization via caveolae/rafts as against clathrin-dependent pathways.

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online data

SUPPLEMENTARY ONLINE DATA

Nuclear translocation and signalling of L1-CAM in human carcinoma cells requires ADAM10 and presenilin/γ-secretase activity

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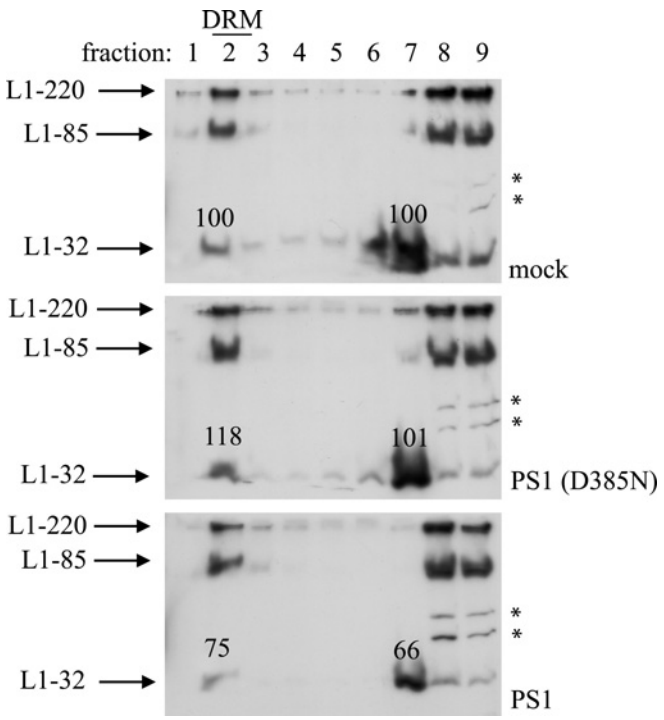

Figure S1 Overexpression of dominant-negative PS1 blocks processing of L1-32

OVMz cells were transfected with wild-type PS1 or a dominant-negative mutant (D385N). After 48 h, cells were lysed in lysis buffer containing Triton X-100 and sucrose-density-gradient centrifugation was carried out as described in the main paper. Numbers indicate relative band intensities. The asterisk (*) indicates non-specific bands.

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