Novel Proteolytic Fragments of the Cell Adhesion Molecule L1, Their Intracellular Trafficking and Functional Roles in the Nervous System of *Mus Musculus* (Linnaeus, 1758)

DISSERTATION

Zur Erlangung der Würde des Doktors der Naturwissenschaften
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vorgelegt von
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To whom it may concern:

David Lutz is submitting his doctoral dissertation in English. The title of his thesis is: 'Novel Proteolytic Fragments of the Cell Adhesion Molecule L1, their Intracellular Trafficking and Functional Roles in the Nervous System of Mus Musculus (Linnaeus, 1758)'.

I hereby certify as a native speaker and molecular biologist that the English language used in this thesis is sufficiently correct for submission.

Kent Duncan, Ph.D.

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Contents

Abstract

Homophilic interactions of the cell adhesion molecule L1 mediate not only mechanical adhesion among cells, but also, importantly, propagate intracellular signals due to connections with the major signalling networks that control most cellular responses. The ability of the cell adhesion molecule L1 to modulate cellular responses has been traditionally viewed as a direct consequence of its adhesive functions. However, after L1-specific stimulation with antibody 557, shedding of L1 occurs and dispossesses this recognition molecule of its adhesiveness as a static cell surface molecule. In fact, under these conditions stimulation of signalling cascades becomes a manifestation of emerging soluble L1 proteolytic fragments, which act as triggers of gene expression. The present work provides novel insights into the proteolytic processing of L1 upon L1-antibody stimulation: triggering of signalling with L1-antibody leads to serine protease-dependent cleavage of full-length L1 at the plasma membrane and generation of a sumoylated transmembrane 70 kDa fragment comprising the intracellular and transmembrane domains and part of the extracellular domain of L1. This fragment travels from the plasma membrane to the late endosomal compartment, where it is released from endosomal membranes into the cytoplasm, and then transported into the nucleus by a pathway that depends on importin and chromatin-modifying protein 1. The 70 kDa L1 fragment is not only transported to the endosomal compartment, but also shortly after generation cleaved by cathepsin E at the plasma membrane. Cathepsin E cleaves the 70 kDa L1 fragment at Glu_{1167} generating a smaller 30 kDa fragment. This 30 kDa L1 fragment is rapidly released into the cytoplasma after generation at the plasma membrane and is then translocated into the nucleus in an importin-dependent manner. Addition of the serine protease

inhibitor aprotinin or mutation of the sumoylation site at Lys_{1172} abolishes L1-stimulated generation and nuclear import of both fragments. Their nuclear import is also impaired after alteration of the nuclear localization signal at Lys_{1147} of L1. Mutation of the putative cleavage site at Glu_{1167} as well as addition of the aspartyl protease inhibitor pepstatin abolishes generation of the 30 kDa L1 fragment. Furthermore, pepstatin inhibits L1-dependent migration of cerebellar neurons from explants, but not neurite outgrowth, indicating that the cathepsin E-mediated cleavage and/or possibly nuclear import of the 30 kDa fragment are necessary for L1-induced neuronal migration. Since aprotinin inhibits L1-stimulated neurite outgrowth, it is very likely that the 70 kDa fragment is involved in regulation of neuritogenesis. Nuclear import of the 70 kDa fragment might activate cellular responses in parallel or in association with phosphorylation-dependent signalling pathways. Alterations in the levels of the 70 kDa fragment during development and in the adult after spinal cord injury or in a mouse model of Alzheimer's disease suggest that this fragment is functionally implicated in development, regeneration, neurodegeneration, and possibly sysnaptic plasticity in the mature nervous system.

Contributions to This Work

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Contributions of the authors to this work are indicated within the figure legends.

Introduction

Adhesion between cells which involves the binding of cell adhesion molecules (CAMs) on adjacent cells regulates fundamental processes such as cell differentiation, cell growth and apoptosis. These processes ensure correct tissue organization in development, and tissue regeneration in the adult. Homophilic interactions between identical CAMs mediate mechanical adhesion among cells but also, importantly, CAMs are able to propagate intracellular signals (Cavallaro and Dejana, 2011).

L1-Family

The cell adhesion molecule L1 is the prototype member of the L1-family of closely related neural adhesion molecules. This family comprises four different members in vertebrates: L1CAM, close homolog of L1 (CHL1), neuro-glia-CAM-related CAM (NrCAM) and neurofascin (Moos et al., 1988; Maness and Schachner, 2007). These molecules regulate neuronal development and networking (Burden-Gulley et al., 1997; Hortsch, 2000; Katidou et al., 2008). The L1 molecule was initially identified in mice (Rathjen and Schachner, 1984) and orthologous or homologous proteins have been detected in human (Wolf et al., 1988), rat (NILE, nerve growth factor-inducible large external glycoprotein) (Lee et al., 1981), chicken (NgCAM, neuron-glia CAM) (Grumet et al., 1984), and Drosophila (neuroglian) (Bieber et al., 1989). Defects in L1 family members are implicated in various neurological diseases of different severity (Kenwrick et al., 2000; Sakurai et al., 2002; Frints et al., 2003; Mathey et al., 2007). Ablation of L1 family members in mice leads to guidance errors in corticospinal and retino-collicular axons, hippocampal CA3 mossy fibers, olfactory neurons, and retinal axons (Maness and Schachner, 2007).

Structure of L1 and Post-translational Modifications

L1 is a 200-220 kDa type-I membrane glycoprotein consisting of six immunoglobulin-like (Ig) domains and five fibronectin-type III repeats (FNIII), followed by a transmembrane region and a highly conserved cytoplasmic tail (Moos et al., 1988). Over the past three decades L1 has been shown to interact with various binding partners in "cis" (at the plasma membrane of one cell, De Angelis et al., 1999) and "trans" (between adjacent cells, Zhao et al., 1998) configuration. L1 can bind to itself homophilically (Su et al., 1998; Freigang et al., 2000; Meijers et al., 2007; Mörtl et al., 2007, Sawaya et al., 2008) or heterophilically to other neural cell adhesion molecules, such as integrins, CD24, neurocan and neuropilin-1. (Schachner, 1997, Brümmendorf et al. 1998; Loers and Schachner, 2007; Herron et al., 2009; Schäfer and Altevogt, 2010) The cytoplasmic tail of L1 interacts with the cytoskeletal proteins ankyrin, actin, spectrin and ERM (ezrin-radixin-moesin) proteins (Bennett and Baines, 2001; McCrea et al., 2009).

A great body of evidence shows that L1 is post-translationally highly modified. For instance, L1 has the potential for glycosylation in all domains. Moreover, various studies have shown that glycosylation affects L1 homophilic interactions (Acheson et al., 1991; Kleene et al., 2001). The ectodomain of L1 is a lectin that can bind to sialic acid (Kleene et al., 2001). Carbohydrates on L1 play a pivotal role in determining the distance between interacting dimers of adhesion molecules, thus modulating homophilic interaction patterns (Wei and Ryu, 2012). L1 is ubiquitinated in its C-terminal part (Schäfer et al., 2010), yet still it remains unknown how ubiquitination and other post-translational modifications modulate the functions of L1.

FUNCTIONS OF L1 IN THE NERVOUS SYSTEM AND TUMORS

During brain development, L1 is involved in several morphogenic events, such as neuron-neuron adhesion, synaptogenesis and synaptic plasticity, glial process formation, neurite outgrowth, fasciculation of axons, axonal guidance and pathfinding, and migration (Keilhauer et al., 1985; Fischer et al., 1986; Chang et al., 1987; Hortsch, 1996; Kamiguchi and Lemmon, 1997; Lee et al., 2008). Recent data has revealed that L1 plays a crucial role in learning and memory (Law et al., 2003), as well as regeneration after trauma (Maness and Schachner, 2007). Several studies implicate L1 in myelination processes and suggest that it mediates the elongation of Schwann cells along the axon and promotes neural cell survival (Lindner et al., 1983; Wood et al., 1990; Conacci-Sorrell et al., 2005). In the central nervous system, L1 is expressed only by post- and pre-mitotic neurons and mainly on non-myelinated axons, whereas in the peripheral nervous system it is expressed by neurons as well as by non-myelinating Schwann cells. L1 is further expressed by hematopoetic (Kowitz et al., 1992; Ebeling et al., 1996; Pancook et al., 1997), kidney (Debiec et al., 1998), pigment, and certain epithelial cells, as well as in a variety of tumor cell lines (Linnemann et al., 1989; Patel et al., 1991; Reid and Hemperly 1992; Katayama et al., 1997), suggesting a potential role of the molecule in non-neural adhesion and migration events.

A large number of neurological disorders have been linked to mutations in the L1 gene, including X-linked hydrocephalus, MASA syndrome (mental retardation, aphasia, shuffling gait, and adducted thumbs), agenesis/dysgenesis of the corpus callosum, X-linked spastic paraplegia, fetal alcohol syndrome and schizophrenia (Kamiguchi et al., 1998; Kurumaji et al., 2001; Itoh et al., 2004, Katidou et al., 2008). More severe consequences are associated with mutations of the extracellular region of L1, which may disrupt adhesion and signalling,

whereas milder symptoms occur with mutations in the cytoplasmic domain, which may alter signalling or interactions with the cytoskeleton (Yamasaki et al., 1997; Kamiguchi et al., 1998).

L1 knockout mice (L1-KO mice) have been generated in two laboratories, and have been extensively examined in order to define the molecular basis of the L1-syndrome (Dahme et al., 1997; Cohen et al., 1998; Fransen et al., 1998). L1-KO mice showed reduced corticospinal tract, abnormal pyramidal decussation (Jakeman et al., 2006), decreased axonal association with non-myelinating Schwann cells, ventricular dilatation, and hypoplasia of the cerebellar vermis (Itoh et al., 2004). Another study reported abnormal morphogenesis of cortical dendrites, showing that pyramidal neurons in layer V exhibited undulating apical dendrites that did not reach layer I, as well as a smaller hippocampus with fewer pyramidal and granule cells and altered distribution of dopaminergic neurons in the brain of L1 null mice (Demyanenko et al., 2001). Reduced size of corpus callosum often appears due to the failure of many callosal axons to cross the midline. These findings reveal a variety of biological roles for L1 that are crucial for correct brain architecture and development.

The study of fundamental L1-mediated events in the nervous system has been approached by a pioneering work in development of antibodies recognizing L1 (Appel et al., 1995; Chen et al., 1999). Generated L1 atibodies have been shown not only to be beneficial for detection of L1, but also for triggering of L1 signalling cascades: antibody 557 and recombinant L1-Fc, which is a hybrid of the extracellular domain of L1 with Fc, have been found to promote survival of neurons and neuritogenesis (Appel et al., 1995; Chen et al., 1999). They have been successfully used as mimetics of homophilic interaction,

which can stimulate L1-mediated cellular responses (Mechtersheimer et al., 2001; Kiefel et al., 2012)

Subsequent research in tumor biology has tremendously extended the view on the importance of L1 in human disease. L1 is overexpressed in many human cancers (ovarian and endometrial carcinoma, pancreatic ductal adenocarcinoma, melanoma, glioblastoma; Kiefel et al., 2012), and L1 expression is mainly associated with poor prognosis, an adhesive phenotype, and advanced tumor stages.

Unlike in neural cells, in tumors L1 rarely promotes static cell-cell adhesion that keeps tumor cells together, but rather induces a motile and invasive phenotype accompanied by aggressive tumor growth, metastasis and chemoresistance (Schäfer and Altevogt, 2010). These fundamental differences and dual mode of action of L1 are not well understood. However, the dual nature of L1 to act not only as a static cell adhesion molecule, but also as an activator of signalling appears to be governed by a process called ectodomain shedding, as described below.

Ectodomain Shedding of L1

Ectodomain shedding has emerged as a key mechanism for regulating the function of cell surface proteins (Arribas and Borroto, 2002). L1 is a type I transmembrane molecule and can undergo ectodomain shedding to release a soluble form into the extracellular space. Soluble L1 stimulates cell migration and is significantly increased in the serum and ascites of ovarian carcinoma patients (Fogel et al., 2003; Maretzky et al., 2005; Bondong et al., 2012), sera of patients with gastrointestinal stromal tumors (Zander et al., 2011), and in cerebrospinal fluid of Alzheimer patients (Strekalova et al., 2006). Full-length

L1 (Fig. 1A) can be proteolytically cleaved within the third FNIII repeat (Fig. 1B) of the ectodomain by the serine proteases plasmin and trypsin or the pro-protein convertase PC5A (Sadoul et al., 1988; Nayeem et al., 1999; Kalus et al., 2003). This particular cleavage site consists of the consensus sequence $_{840}$RKHSKR$_{845}$ which resembles the recognition motif for pro-protein convertases, (R/K)X0,2,4,6(K/R) (Kalus et al., 2003). This cleavage generates an 80 kDa (Fig. 1B) transmembrane and a soluble extracellular 140 kDa fragment (Fig. 1C). Soluble 180 kDa (Fig. 1E) or 50 kDa (Fig. 1G) and 32 kDa membrane-bound (Fig. 1D) fragments result from membrane-proximal shedding of the membrane-spanning 200 kDa or 80 kDa L1 form, respectively, by members of the ADAM (a disintegrin and metalloproteinase) family, such as ADAM10 (Mechtersheimer et al., 2001; Maretzky et al., 2005; Riedle et al., 2009). Biochemical evidence suggests that the smaller non-soluble cleavage product of L1 is retained at the membrane, as it can be detected by cell surface iodination (Hubbe et al., 1993; Beer et al., 1999). The membrane proximal cleavage by ADAMs which releases the entire ectodomain of L1 (Beer et al., 1999) occurs in tumor cell lines as well as in the developing mouse brain (Gutwein et al., 2000; Mechtersheimer et al., 2001). The ADAM10-mediated 32 kDa L1 proteolytic product is further cleaved by presenilin/γ-secretase (Fig. 1F) resulting in the generation of an intracellular L1 fragment of approximately 28 kDa (Riedle et al., 2009). This fragment is found in the nucleus, where it induces nuclear signalling and modulates gene expression (Riedle et al., 2009).

Shedded soluble ectodomains of L1 are functionally active and can promote cell migration (Mechtersheimer et al., 2001; Yang et al., 2009), protect cells from apoptosis (Stoeck et al., 2007; Sebens Müerköster et al., 2007), stimulate cell survival (Voura et al., 2001; Nishimune et al., 2005) and act as pro-angiogenic factors (Hall and Hubbell, 2004; Friedli et al., 2009). L1 and

ADAM10 can co-operate to promote cancer cell motility (Gutwein et al., 2005; Kiefel et al., 2012). Soluble L1 in ascites is a proven marker for poor progression-free survival and chemoresistance (Zander et al., 2011; Bodong et al., 2012).

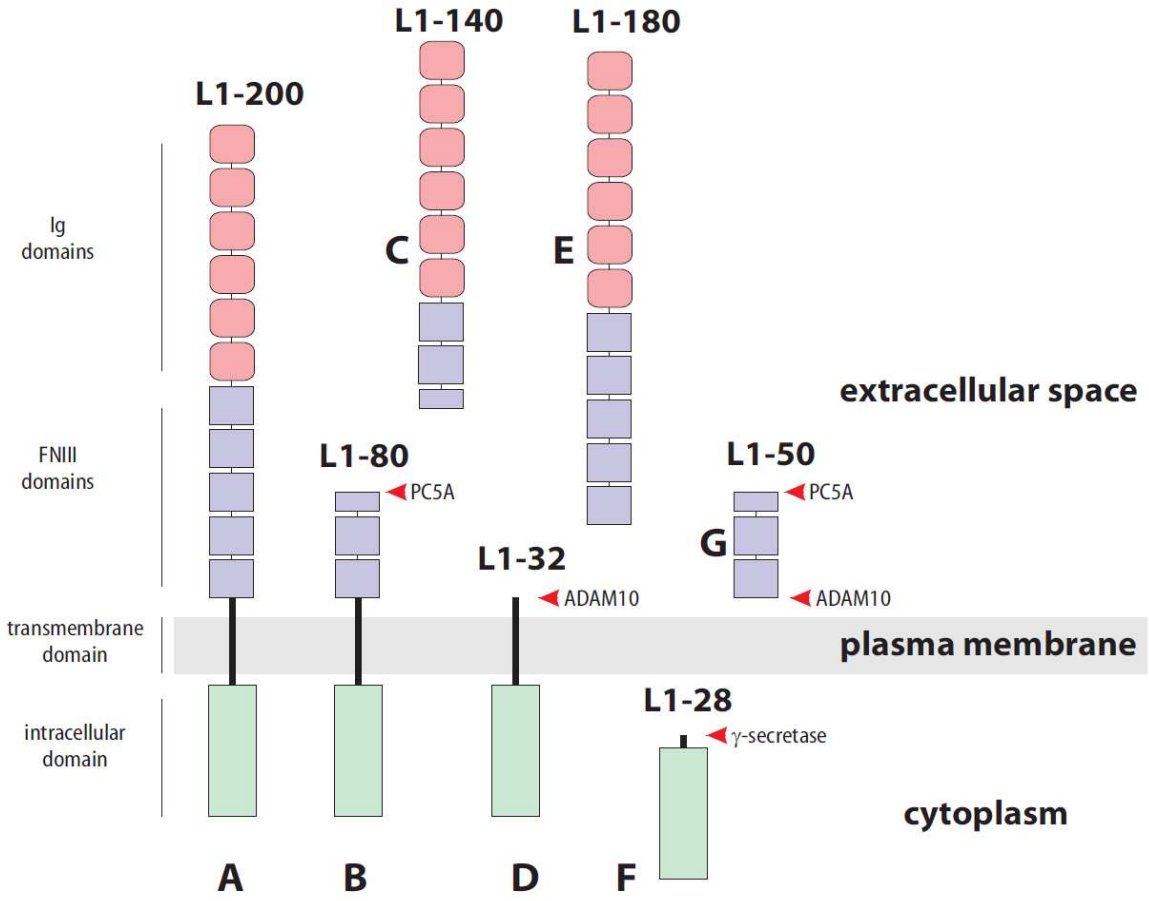

FIGURE 1. **Ectodomain shedding of L1.** *A*, Full-length L1 (L1-200) consists of six Ig-like domains, five FNIII repeats, a transmembrane and an intracellular domain. *B,C*, PC5A cleaves L1-200 within the 3rd FN repeat to generate a membrane-bound 80 kDa (L1-80) and a soluble 140 kDa (L1-140) fragment. *D,E,G*, L1-200 and L1-80 are substrates of the ADAM10 protease, which generates a membrane-bound 32 kDa (L1-32) and a soluble 180 kDa (L1-180) or a soluble 50 kDa (L1-50) fragment, respectively. *F*, L1-32 can be processed by the γ-secretase to an intracellular 28 kDa (L1-28) fragment.

The transmembrane L1 remnants that result from ectodomain shedding appear to undergo regulated intramembrane proteolysis (RIP; Riedle et al., 2009).

RIP is an essential step in a variety of signalling pathways (Ebinu and Yanker, 2002; Landman and Kim, 2004). Since smaller L1 fragments are found in the nucleus (Riedle et al., 2009), these fragments have been suggested to regulate signalling and gene expression: if RIP is abolished, L1-mediated gene regulation and signalling are abrogated as well (Riedle et al., 2009; Kiefel et al., 2012). Furthermore, regulation of several cancer-related genes, such as the transcription factor homebox A9 (HOX-A9) and activating enhancer-binding protein 2α (AP2α), as well as the tumor suppressor CRABPII, regulator of apoptosis IER3, cathepsins, and β3-integrin have all been shown to depend on cleavage of L1 and its cytoplasmic part (Riedle et al. 2009; Gast et al., 2008).

How exactly these soluble ectodomain fragments and membrane-bound remnants of L1 are able to perform all of these functions is still barely understood. However, cleavage in the extracellular domain confers L1 new properties. Thus, it is likely that the remaining intracellular tail or the soluble ectodomain may still interact with signalling partners and promote direct or indirect intracellular signalling. How and when this occurs is unclear. Moreover, very little is known about the fate of the proteolytic fragments of L1 after ectodomain shedding, their intracellular distribution, and functional roles in the choreography of cellular responses to L1-mediated stimuli.

Aim of the Thesis

The aim of this thesis was to investigate novel proteolytic fragments of the neuronal cell adhesion molecule L1 generated upon L1 specific stimulation, their intracellular trafficking within neural cells and functional roles in the nervous system.

To achieve these goals the project has been split into following steps:

1. Identification of novel proteolytic L1 fragments in cultured murine cerebellar neurons after L1-specific stimulation.
2. Investigation of trafficking and nuclear import of the identified fragments.
3. Investigation of post-translational modifications required for L1-processing on the plasma membrane.
4. Identification of cleavage sites on L1 and proteases generating the fragments.
5. Assessment of functional roles of the novel proteolytic L1 fragments in the mouse nervous system.

Materials and Methods

Animals

C57BL/6J mice were used as wild-type mice. L1-deficient mice (Rolf et al., 2001) were kept as heterozygous breeding pairs maintained on a mixed genetic background (129SVJ x C57BL/6 x Black Swiss). Generation and breeding of the L1-deficient mice have been described (Guseva et al., 2009). Additionally, 7-month-old transgenic APPPS1-21 (C57BL/6J-TgN; Thy1-$APP_{KM670/671NL}$; Thy1-$PS1_{L166P}$) mice were obtained from a breeding colony at University of Tübingen, Germany. These mice co-express mutated human amyloid precursor protein (Swedish double mutation) and a mutated presenilin-1 under a neuron-specific murine Thy-1 promoter element on a C57BL/6J background (Radde et al., 2006). Animals were housed at 25°C on a 12-h light/12-h dark cycle with *ad libitum* access to food and water. All animal experiments were approved by the local authorities of the State of Hamburg (animal permit numbers ORG 535 and G09/098) and conform to the guidelines set by the European Union.

Reagents and Antibodies

Polyclonal antibodies to mouse L1 that react with the extracellular domain and rat monoclonal antibodies 557 and 555 against distinct epitopes at the N terminus of the third FNIII domain or between the second and third FNIII domains, respectively, have been described (Appel et al., 1995). Monoclonal mouse L1 antibody 172-R against the intracellular domain of L1 was obtained from HISS Diagnostics. All secondary antibodies were obtained from Dianova. Antibodies against importin-α, importin-β, histone H1, and heterochromatin-associated protein 1-γ (HP1γ) were purchased from Sigma-Aldrich, Abcam, MBL International, Millipore, and Cell Signaling Technology, respectively.

Antibodies against protein-disulfide isomerase, actin, apoptosis-linked gene-2-interacting protein X (Alix), tumor susceptibility gene 101 (Tsg101), vacuolar protein sorting-associated protein 4 (Vps4), chromatin-modifying protein 1 (CHMP1), cathepsin E and cathepsin D were obtained from Santa Cruz Biotechnology. Pan-ubiquitin and pan-small ubiquitin-like modifier (SUMO) antibodies were obtained from Santa Cruz Biotechnology or Abgent. Mouse L1-Fc was prepared as described (Chen et al., 1999). GAPDH antibody was purchased from Calbiochem. βIII-tubulin antibody was from Covance. Cathepsin D siRNA, cathepsin E siRNA, control siRNA, siRNA transfection reagent and siRNA transfection medium were obtained from Santa Cruz Biotechnology. Poly-L-lysine (PLL, molecular weight 70,000 – 150,000) was from Sigma. Matrigel was purchased from BD Biosciences. DAPT (γ-secretase inhibitor), GM6001 (matrix-metalloproteinase inhbitor) and MG132 (proteasome inhibitor) were from Calbiochem. Aprotinin, leupeptin and pepstatin were from Sigma-Aldrich. Vectors encoding GFP-sumo-1, GFP-sumo-2, and GFP-sumo-3 were kindly provided by Hans Will (Heinrich-Pette-Institut and Leibniz Institute for Experimental Virology, Hamburg, Germany). OptiPrep was from Axis-Shield.

SITE-DIRECTED MUTAGENESIS OF L1

To disrupt the nuclear localization site Lys_{1147} (exchange of KRSK to RRSK), the sumoylation site Lys_{1172} (exchange of MKDE to MRDE), or concomitantly the nuclear localization signal and the sumoylation site Lys_{1235} (exchange of GKKE to GRKE) the primer pairs up1 (5′-CTC ATC CTC TGC TTC ATC AGA CGC AGC AAG GGT GGC AAA TAC-3′) and down1 (5′-A TTT GCC ACC CTT GCT GCG TCT GAT GAA GCA GAG GAT GAG CA-3′), up2 (5′-TA GAT TCC GAG GCC CGG CCC ATG AGA GAC GAG ACC TTC GGC GA-3′) and down2 (5′-T GTA

CTC GCC GAA GGT CTC GTC TCT CAT GGG CCG GGC CTC GGA AT-3′), or up3 (5′-T TTC ATC GGC CAG TAC AGT GGC AGG AAA GAG AAG GAG GCA GCA-3′) and down3 (5′-T GCC TCC TGC TGC CTC CTT CTC TTT CCT GCC ACT GTA CTG GCC GA-3′) (underlined letters indicate the exchanges), respectively, were used in GENEART® Site-Directed Mutagenesis System (Invitrogen). Site-directed mutagenesis was performed to disrupt the putative cathepsin E cleavage site by exchanging the glutamic acid residue at position 1167 by a glutamine residue ($E_{1167}Q$): Forward primer (5'-GAC ACT CAG GTA GAT TCC CAG GCC CGG CCC ATG AAA GAC GAG ACC-3') and reverse primer (5'-GTC TTT CAT GGG CCG GGC CTG GGA ATC TAC CTG AGT GTC CTC CTT-3') (underlined letters indicate the exchange).

Transfection of HEK Cells

HEK293TN (BioCat) cells were plated in 6-well plates (Nunc) at a density of 2×10^5 cells/well and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 4.5 mg/ml glucose, 10% fetal calf serum, and 100 units/ml penicillin/streptomycin for 24 h; and then transfected using 6 µl of TurboFect (Fermentas) and 4 µg vector DNA in 200 µl of serum-free DMEM according to the manufacturer's instructions.

Cultures and Treatments of Cerebellar Neurons and SH-SY5Y Cells

Cerebellar neurons were cultured as described (Kleene et al., 2010). SH-SY5Y (ATCC number CRL-2266™) cells were cultured in 6-well plates (Nunc) for 24 h in high glucose (4.5 g/liter) DMEM supplemented with 10% fetal calf serum, 1 mM sodium pyruvate (PAA Laboratories), 2 mM L-glutamine (Invitrogen), and 100 units/ml penicillin and streptomycin (Invitrogen). Cells

were maintained at 37°C, 5% CO_2, and 90% humidity. SH-SY5Y cells or freshly dissociated cerebellar neurons were seeded into 6-well plates (Nunc) at a density of 2×10^6 cells/well, maintained for 24 h, and serum-deprived for 5 h. Cells were then treated with rabbit polyclonal L1 antibody or rabbit non-immune control serum (corresponding to 5 μg of IgG/ml; Jackson ImmunoResearch Laboratories), with monoclonal L1 antibody 557 or rat non-immune control IgG (50 μg/ml; Jackson ImmunoResearch Laboratories), or with L1-Fc or Fc (10 μg/ml) in the absence or presence of 1 μM aprotinin for 1 h at 37°C.

Transient siRNA Transfection of Cerebellar Neurons

For each transfection, 6 μl of siRNA transfection reagent were mixed with 100 μl of siRNA transfection medium and added to a mixture of 4 μg siRNA and 100 μl of siRNA transfection medium. After incubation at room temperature for 45 min, the transfection samples were added to 1×10^6 cultured cerebellar neurons. After incubation at 37°C and 5% CO_2 for 6 h, transfected cells were incubated in 1 ml of culture medium (Neurobasal A (Gibco) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 4 nM L-tyroxine, 1× B27 supplement, 10 μg/ml BSA, 100 μg/ml transferrin holo, 10 μg/ml insulin, 10 ng/ml selenium, 50 units/ml penicillin and 50 units/ml streptomycin) for 18-24 h followed by incubation in serum-free culuture medium for 5 h.

Transient Co-transfection of HEK Cells with L1, mutant L1, cathepsin E and cathepsin E-siRNA for Scratch Assay

HEK293TN (BioCat) cells were grown on glass coverslips in 48-well plates and maintained in cell culture medium (DMEM supplemented with 2 mM L-glutamine, 4.5 mg/ml glucose, 10% fetal calf serum, and 100 units/ml of

penicillin/streptomycin) until reaching a confluence of 80%. For each transfection, 1 μg cathepsin E siRNA or 1 μg DNA (L1, L1 mutated at E_{1167} or cathepsin E) were mixed with 2 μl of FuGENE® HD (Promega) and 100 μl of DMEM medium supplemented with 100 units/ml of penicillin/streptomycin. After incubation at room temperature for 15 min, the transfection samples were added to the cells. After incubation at 37°C and 5% CO_2 overnight, a scratch assay was performed as decribed (Kuang et al., 2009) with slight modifications. Briefly, the confluent monolayer of HEK cells was scratched with a sterile white 10 μl plastic tip, producing a cell-free cleft of 650 μm width. After scratching, cells were immediately washed with DMEM medium and then incubated with or without antibody 557 (final concentration of 25 μg/ml) at 37°C and 5% CO_2 for 20 h. All further steps were performed at room temperature. For assessment of gap closure after 20 h, the glass coverslips were fixed in 4% paraformaldehyde for 20 min and incubated in 1% BSA/PBS blocking solution for 30 min. Immunostaining with antibody 557 (dilution 1:250) for 2 h and secondary Cy2 anti-rat antibody (dilution 1:250) for 1 h was performed. Glass coverslips were mounted in Fluromount medium containing DAPI (Carl Roth). Fluorescent confocal images were taken on the OLYMPUS F1000 imaging system (Olympus) using a 10× objective and gap width was measured with ImageJ software. Six coverslips from two independent experiments were analyzed per condition. Two images per coverslip were taken and 10 gap widths from each image were measured. Differences between the groups were statistically evaluated using one-way-ANOVA with Tukey's Multiple Comparison Test.

Cell Surface Biotinylation of SH-SY5Y Cells

SH-SY5Y cells were grown in twenty 15-cm dishes until reaching 70–80% confluence and incubated with serum-free medium for 8–12 h. Cells were

washed three times with PBS^{2+} (phosphate-buffered saline, pH 7.3 (PBS) supplemented with 0.5 mM $CaCl_2$, 2 mM $MgCl_2$) and incubated for 30 min at room temperature with 0.5 mg/ml sulfo-NHS-LS-biotin (Pierce) in PBS^{2+} followed by two washing steps with 100 mM glycine at room temperature. Cells were washed with PBS^{2+} and treated with L1 (1 μg/ml) or control antibodies (1 μg/ml) at 37°C for 1 h. After removal of the culture medium, cells were washed twice with PBS, resuspended in hypotonic buffer (100 mM HEPES, pH 7.8, 20 mM KCl, 2 mM EGTA) containing protease inhibitors (Roche Diagnostics) - 1 tablet/50 ml added just before the experiment, and harvested using a rubber policeman. Cells were centrifuged for 5 min at 600 × *g* and 4°C. The volume of the pellet was measured, 2 volumes of isotonic buffer (50 mM HEPES, pH 7.8, 0.25 M sucrose, 20 mM KCl, protease inhibitors) were added to the pellet, and the pellet was homogenized using a Dounce homogenizer and passed through a 27-gauge needle several times at 4°C.

Subcellular Fractionation

Brains of 2-day-old mice or SH-SY5Y cells were homogenized in homogenization buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.4). Cell or brain homogenate was incubated for 1 h at 37°C to allow proteolysis and increase the amount of L1 fragments. After centrifugation at 1,000 × *g* for 10 min at 4°C, the 1,000 × *g* pellet containing nuclei was saved as the nuclear fraction, and the resulting 1,000 × *g* postnuclear supernatant was centrifuged at 17,000 × *g* for 20 min at 4°C. All subsequent steps were carried out at 4°C.

The 17,000 × *g* supernatant was further centrifuged at 100,000 × *g* for 45 min. The 100,000 × *g* pellet was taken as the microsomal fraction, and the supernatant containing soluble proteins was taken as the cytoplasmic fraction. For the isolation of the plasma membrane fraction, the 17,000 × *g* pellet was

washed once with homogenization buffer and subjected to hypotonic shock by resuspending the pellet in 9 volumes of ice-cold H_2O containing protease inhibitors (Roche Diagnostics). The resuspended fraction was adjusted to 5 mM Tris-HCl by adding 1 M Tris-HCl (pH 7.5), stirred for 30 min, and centrifuged at 25,000 × *g* for 20 min. The pellet was homogenized in homogenization buffer using a Dounce homogenizer and by passing through a 27-gauge needle and loaded onto a discontinuous sucrose gradient, which consisted of 0.8, 1.0, and 1.2 M sucrose/PBS. After centrifugation at 150,000 × *g* for 2 h, the interphase between 1.0 and 1.2 M sucrose, which contains plasma membranes, was collected and diluted with 2 volumes of homogenization buffer.

For the isolation of endosomes, the 100,000 × *g* microsomal pellet was resuspended in 2 M sucrose and applied to a gradient containing 0.25, 0.8, 1.15, and 1.3 M sucrose/PBS. After centrifugation at 100,000 × *g* for 2 h, the fraction at the interphase between 0.8 and 1.15 M sucrose, which contains endosomes, was collected.

For the isolation of nuclei, the 1,000 × *g* nuclear pellet was homogenized in homogenization buffer and applied to a gradient of 35, 30, and 25% OptiPrep/PBS and centrifuged at 10,000 × *g* for 20 min. Nuclei were collected from the 30/35% interphase, diluted with 2 volumes of homogenization buffer, and centrifuged at 1,000 × *g* for 20 min. The pellet was again resuspended in homogenization buffer and centrifuged at 1,000 × *g* for 10 min. The nuclear pellet was then resuspended in 200 μl of extraction buffer (10 mM HEPES, 10 mM KCl, 2 mM $MgCl_2$, 500 mM NaCl, 25% glycerol, pH 7.5, protease inhibitor mixture) and incubated on ice for 30 min. After centrifugation at 10,000 × *g* for 5 min, the supernatant was collected, the pellet was resuspended in SDS sample buffer (see below) and passed through a

27-gauge needle. The supernatant was designated the "nuclear protein extract," and the pellet was designated "insoluble nuclear protein."

For the isolation of exosomes, cell culture supernatants were collected and centrifuged for 10 min at 1,000 × *g* and for 15 min at 17,000 × *g* to remove cells and cellular debris. Exosomes were collected by centrifuging the resulting cell-free supernatant at 100,000 × *g* for 1 h.

For the isolation of the endoplasmic reticulum (ER), an ER isolation kit (Sigma) was used following the manufacturer's instructions. Briefly, cells were centrifuged at 1,000 × *g*, resuspended in hypotonic buffer containing the protease inhibitors, incubated for 20 min on ice, and centrifuged at 600 × *g* for 5 min. The pellet was suspended in 2 volumes of isotonic buffer containing protease inhibitors and homogenized with a Dounce homogenizer and by passing through a 27-gauge needle. The samples were centrifuged at 1,000 × *g* for 10 min, and the resulting supernatants were centrifuged at 12,000 × *g* for 15 min followed by centrifugation of the resulting supernatants at 100,000 × *g* for 1 h. The pellets were homogenized in homogenization buffer, adjusted to 20% OptiPrep/PBS, layered between 30 and 15% OptiPrep, and centrifuged at 150,000 × *g* for 3 h. The material from the 15/20% interphase containing smooth ER (SER) and the material from the 20/30% interphase containing rough ER (RER) was collected. The Qproteome Nuclear Protein kit (Qiagen), Subcellular Protein Fractionation kit (ThermoScientific), and ExoQuick-TC (System Biosciences) were used for subcellular fractionation according to the manufacturer's instructions.

WESTERN BLOT ANALYSIS, STREPTAVIDIN PULLDOWN, AND IMMUNOPRECIPITATION

Western blot analysis has been described in detail (Makhina et al., 2009). For isolation of biotinylated proteins after cell surface biotinylation, streptavidin-

conjugated magnetic beads (Pierce) were incubated with cell lysates or cellular subfractions overnight at 4°C. After washing the beads, biotinylated proteins bound to the streptavidin beads were eluted by boiling the beads in SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.02% bromphenol blue) for 5 min at 95°C. For immunoprecipitation, samples were resuspended in lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, pH 7.6) and subjected to preclearing and immunoprecipitation using Protein A/G-Agarose Plus (Santa Cruz Biotechnology). Beads were then washed with PBS and boiled in SDS sample buffer for 5 min at 95°C.

Retrotranslocation and Nuclear Import Assay

The endosomal or ER fractions were resuspended in homogenization buffer and incubated with the cytoplasmic fraction or translocation buffer (10 mM HEPES, 40 mM magnesium acetate, pH 7.2, 1 mM DTT, 0.1 mM PMSF) in the presence or absence of 3 μg antibodies for 60 min at 4°C. The samples were then centrifuged at 100,000 × *g* for 20 min at 4°C. The pellets and supernatants were collected, and streptavidin-coupled magnetic beads (Invitrogen) were used to isolate the biotinylated proteins. For the analysis of nuclear import, nuclei isolated from SH-SY5Y cells or from mouse brain were incubated in the absence or presence of 3 μg antibodies in nuclear translocation buffer (25 mM HEPES, pH 7.4, 12.5 mM KCl, 2.5 mM $MgCl_2$, 1.25 mM $CaCl_2$, 0.1 mM ATP), with the buffer used for the isolation of nuclei, or with the cytoplasmic fraction isolated from SH-SY5Y cells after cell surface biotinylation and stimulation of L1 functions or from mouse brain homogenates after incubation at 37°C for 1 h. Streptavidin-coupled magnetic beads were used to isolate biotinylated proteins.

Neurite Outgrowth, Immunocytochemistry of Cerebellar Neurons and Image Acquisition

Cerebellar neurons were seeded on poly-L-lysine-coated glass coverslips and maintained in serum-free medium as described (Makhina et al., 2009). Antibody 557 or control rat antibody (50 μg/ml) and aprotinin (0.042 trypsin inhibitory units) were added 2 h after cell seeding. After 24 hours, cells were fixed in 2.5% glutaraldehyde in Neurobasal A for 60 min at room temperature, stained with 1% toluidine blue and 1% methylene blue in 1% borax (pH 7.4) and neurite outgrowth was analyzed by measuring the total length of neurites in a AxioVert135 microscope equipped with AxioVision software 4.7 (Carl Zeiss). Approximately 100 cells from two coverlsips per condition were counted. Differences between the groups were statistically evaluated using one-way ANOVA with Tukey's Multiple Comparison Test. For immunostaining, cultured neurons were washed with ice-cold PBS, fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, washed, permeabilized with 1% Triton X-100 in PBS for 5 min, and blocked in 1% BSA in PBS for 30 min at 4°C. Primary antibodies in blocking solution (dilution 1:1000) were incubated overnight at 4°C, and Cy2- or Cy3-conjugated secondary antibodies (dilution 1:250) were incubated for 1 h in 1% BSA in PBS at room temperature under exclusion of light. To counterstain nuclei, DRAQ5™ (Biostatus Ltd.) was diluted 1:1,000 in PBS and applied for 15 min at 4°C. Coverslips were embedded in Fluoromount G (SouthernBiotech). Images of neurons were acquired using an Axiophot 2 microscope (Carl Zeiss) equipped with a digital camera (AxioCam HRc), AxioVision software (version 3.1), and a Plan-Neofluar 40× objective (numerical aperture, 0.75) or using a confocal laser-scanning microscope (LSM510, Carl Zeiss), LSM510 software (version 3), and an oil Plan-Neofluar 40× objective (numerical aperture, 1.3) at 3× digital zoom. For quantification

of the fluorescence intensity of nuclear area, one image per cell at the level of the largest nuclear area from stacks of images 1 μm apart was used to measure fluorescence intensity in the area using ImageJ software. Area fluorescence intensity of nuclei of 10 cells per group was determined, and statistical comparisons between the groups were performed using Student's *t* test.

CEREBELLAR EXPLANTS

Explant cultures from mouse cerebella were prepared as described (Kalus et al., 2003). Briefly, cerebella from 6- to 8-day-old C57BL/6J mice were passed through a Nitrex net with a pore width of 300 μm. The small tissue pieces were washed with Hanks' balanced salt solution and culture medium (Neurobasal A (Gibco) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 4 nM L-tyroxine, 1× B27 supplement, 10 μg/ml BSA, 100 μg/ml transferrin holo, 10 μg/ml insulin, 10 ng/ml selenium, 50 units/ml penicillin and 50 units/ml streptomycin). The explants were plated onto PLL-coated glass coverslips and maintained in serum-containing culture medium for 16 h. After this, 1 ml of serum-free culture medium containing protease inhibitors (1 μM aprotinin, 1 μM leupeptin or 1 μM pepstatin) was added to the explants. After 24 h, the explants were fixed with 2.5% glutaraldehyde in Neurobasal A medium for 60 min and stained with 1% toluidine blue and 1% methylene blue in 1% borax (pH 7.4). The effect of the different protease inhibitors on neurite outgrowth and neuronal cell migration was analyzed and quantified by measuring the length of all neurites per explant and the number of cells in distinct distance intervals for three explants using AxioVision 4.7 software (Kontron, Zeiss, Germany). Differences between the groups were statistically evaluated using oneway-ANOVA with Tukey's Multiple Comparison Test.

Alternatively, explants were maintained in Matrigel diluted 1:3 in serum-free culture medium. Explants were then seeded droplet-wise on Matrigel-coated PLL glass coverslips and incubated at 37°C in a hymidified 5% CO_2 atmosphere for ~1 h. Thereafter, serum-free culture medium containing monoclonal L1 antibody 557 with and without inhibitors (1 μM aprotinin or 1 μM pepstatin) was added to the explants for additional 24 h. The explants were fixed with 4% paraformaldehyde, stained with βIII-tubulin (dilution 1:1,000 in PBS) at 4°C overnight. Cy2-donkey anti-rabbit secondary antibody (dilution 1:250 in PBS) was applied at room temperature for 1 h. After washing, the explants were mounted on glass coverslips in Fluromount medium containing DAPI (Carl Roth). Fluorescent confocal images were taken on an OLYMPUS F1000 imaging system (Olympus).

Spinal Cord Injury

Lower thoracic compression spinal cord injury was performed on 3-month-old C57BL/6 female mice as described (Jakovcevski et al., 2007; Mehanna et al., 2010). Briefly, mice were anesthetized by intraperitoneal injection of ketamine and xylazine (100 mg of Ketanest (Pfizer) and 5 mg of Rompun (Bayer)/kg of body weight). Laminectomy was performed at the T7–T9 level with mouse laminectomy forceps (Fine Science Tools). An electromagnetic compression device (Curtis et al., 1993) was used to elicit injury by compressing the delaminated spinal cord for 1 s by a time-controlled 12-V (maximum voltage) current flow through the device. After surgery, mice were allowed to recover at 35°C and were provided with water and food *ad libitum*. Seven days after spinal cord injury mice were sacrificed, and 5-mm-long segments from individual spinal cords were dissected from the part of the spinal cord 5 mm rostral to the injury site. Samples were mechanically dissociated in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic

acid (EDTA), 1mM ethylene glycol tetraacetic acid (EGTA), 1% Nonidet P-40, 1× protease inhibitor cocktail) using a Dounce homogenizer and processed for Western blot analysis as described before.

Results

Transmembrane L1 Fragment Comprising Part of the Ectodomain Appears upon L1 Stimulation and Translocates into the Nucleus

It was first necessary to investigate whether stimulation of L1, which is on the surface of neurons, would not only promote neurite ourgowth and neuronal survival, but also the generation and nuclear import of proteolytic L1 fragments as well. The monoclonal L1 antibody 557, which is known to trigger L1-dependent cellular responses, such as neurite outgrowth (Appel et al., 1995), was therefore used to stimulate neurons. Non-nuclear and nuclear fractions from cultured cerebellar neurons were tested for the presence of L1 fragments by Western blot analysis with the L1 antibody 172-R, which is directed against an epitope within the intracellular domain of L1. The presence of a fragment with an apparent molecular weight of approximately 70 kDa was observed mainly in the fraction containing soluble nuclear proteins after application of L1 antibody 557 for 1 hour (Fig. 2A). This fragment was not detectable in fractions from mock-treated cells or cells treated with a non-immune control antibody (Fig. 2A).

Since the calculated molecular weight of the intracellular domain of L1 is approximately 12 kDa, the nuclear 70 kDa fragment was assumed to comprise not only the intracellular domain, but also the transmembrane domain and part of the extracellular domain of L1. To test this, the antibody 557 directed against an epitope at the N-terminus of the third FNIII domain was used for Western blot analysis of the non-nuclear and nuclear fractions. The 70 kDa L1 fragment was found predominantly in the soluble nuclear protein fraction (Fig. 2B). As antibody 557 could recognize this fragment, it must contain the entire fourth and fifth FNIII domains and at least most of the third FNIII domain.

After longer exposure, significant amounts of the 70 kDa fragment were also detectable in the non-nuclear fraction and in the fraction containing chromatin-associated proteins, while the fragment was hardly detectable in the non-nuclear and nuclear fractions of neurons incubated with non-immune control antibody (Fig. 2B). Cell culture supernatants of neurons treated with antibody 557 contained an L1 fragment with an apparent molecular weight of approximately 135 kDa that could be detected with a polyclonal L1 antibody directed against the whole extracellular domain of L1. This fragment was not seen when neurons were treated with a non-immune control antibody or when the 557 antibody was used for detection in Western blot analysis (Fig. 2C). These observations indicate that generation of the transmembrane 70 kDa L1 fragment is accompanied by generation of a corresponding soluble extracellular 135 kDa fragment. The cell culture supernatants from L1-deficient cerebellar neurons stimulated with antibody 557 similarly were not positive for 135 kDa fragment (Fig. 2C). Their non-nuclear and nuclear fractions were not positive for the 70 kDa fragment (Fig. 2D). This confirms that both fragments are L1-specific.

The finding that the 70 kDa fragment, but not the 135 kDa fragment, was detected by antibody 557 shows that the 70 kDa fragment is distinct from the previously described transmembrane 80 kDa fragment (Sadoul et al., 1988; Kalus et al., 2003), which lacks the epitope recognized by antibody 557 (Appel et al., 1995) and that the soluble extracellular 135 kDa fragment differs from the well-characterized extracellular 140 kDa fragment (Sadoul et al., 1988; Kalus et al., 2003) which is recognized by the antibody 557. Since antibody 557 recognizes the third FNIII domain in L1 (Appel et al., 1995), the findings above would suggest, that the 80 kDa and the 135 kDa fragments are lacking this complete domain – so that they cannot be recognized by this antibody. In

contrast, the 70 kDa and 140 kDa L1 fragments contain this domain and can be thus detected by antibody 557. In conclusion, the cleavage site on L1 for generating the 70 kDa fragment must be located N-terminally from the third FNIII domain. Furthermore, the combined results show that stimulation of cultured neurons with L1 antibody leads to the generation of a soluble extracellular 135 kDa fragment and a 70 kDa transmembrane fragment which is translocated to the nucleus.

Since L1-Fc also triggers L1 functions, such as neurite outgrowth (Chen et al., 1999; Loers et al., 2005; Lieberoth et al., 2009; Makhina et al., 2009), it was tested if L1-Fc treatment also induces nuclear import of the 70 kDa fragment. Levels of the 70 kDa fragment in the soluble nuclear protein fraction and of the 135 kDa fragment in the cell-free cell culture supernatant of cerebellar neurons were increased upon application of L1-Fc compared to Fc treatment (Fig. 2E). This result shows that treatment with L1-Fc promotes generation of the 70 and 135 kDa fragments in a similar way as treatment with antibody 557, substantiating further that stimulation of L1 signaling leads to generation of a soluble extracellular 135 kDa fragment and a corresponding 70 kDa transmembrane fragment. In L1-KO mice no L1 and L1 fragments are present.

To verify the nuclear localization of L1 fragments, immunostainings of cerebellar neurons were performed with L1 antibodies 172-R and 555 which are directed against an intracellular epitope and an epitope between the second and third FNIII domain, respectively. A faint nuclear L1 immunoreactivity was observed with these two antibodies in non-stimulated cells, while a pronounced increase in nuclear L1 immunoreactivity was observed upon stimulation with antibody 557 (Fig. 2F). Quantification of the area fluorescence intensity within nuclei indicated that 172-R and 555

immunoreactivities were, respectively, 1.6±0.28 ($p<0.01$) and 3.7±0.6 ($p<0.0018$) times higher in nuclei of stimulated versus non-stimulated neurons. This result confirms that L1 stimulation leads to nuclear import of the 70 kDa fragment.

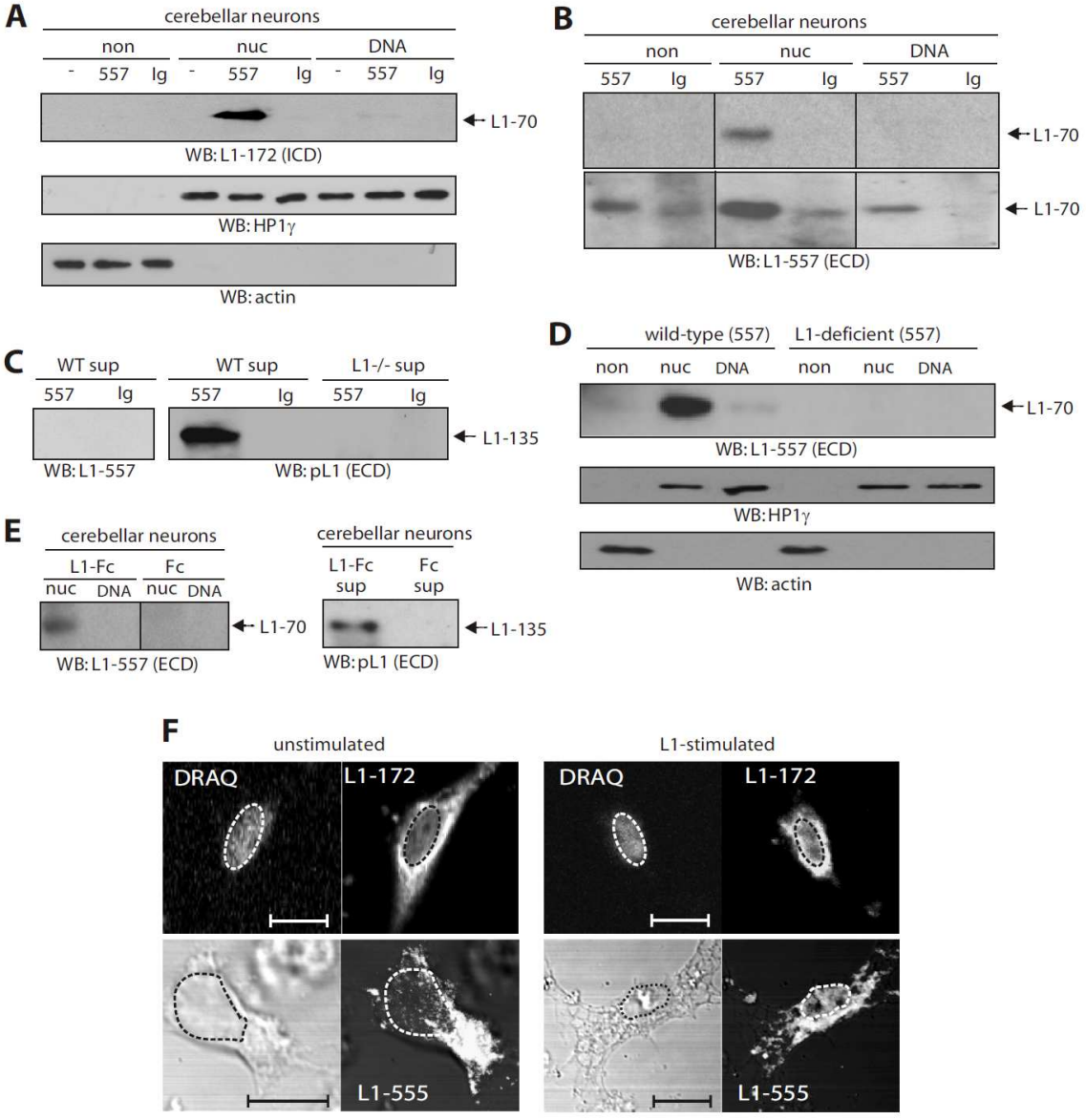

FIGURE 2. **L1-specific stimulation leads to generation and nuclear import of a transmembrane 70 kDa L1 fragment**. *A-F*, cerebellar neurons from wild-type or L1-deficient mice were mock-treated, treated with L1 antibody 557 or non-immune rat antibody (Ig), or treated with Fc or L1-Fc. *A-E*, non-nuclear fraction (non) (*A,B,D*), nuclear fractions containing nucleoplasmic (nuc) or chromatin-associated proteins (DNA)(*A,B,D,E*), and the cell culture supernatant (sup) (*C,E*) were isolated and subjected to Western blot (WB) analysis using antibody 172-R (L1-172) directed against an epitope in the intracellular domain (ICD) (*A*), antibody 557 (L1-557) (*B-E*), or polyclonal L1 antibody

(pL1) (*C,E*) against epitopes in the extracellular domain (ECD). The different L1 fragments seen on a representative blot after different exposure times are indicated. Identical amounts of protein were loaded, and the HP1γ and actin antibodies were used in Western blot analysis to control loading of non-nuclear or nuclear proteins (A and D). Lanes not adjacent to each other but from the same blot are indicated by dividing lines. The experiments were performed four times (*A,B*) or two times (*C–E*) with identical results. *F*, neurons treated with control antibody (unstimulated) or with antibody 557 (L1-stimulated) were subjected to permeabilization and immunostaining with the antibody 172-R or 555, which is directed against an epitope between the extracellular second and third FNIII domains. Immunostaining by antibody 172-R (L1-172), counterstaining of the nucleus (DRAQ), the phase-contrast image, and the immunostaining by antibody 555 (L1-555) of representative neurons are shown. The results were reproduced in two independent experiments. The bar represents 5 μm, and the nuclei are depicted by dashed lines. The experiments (*A,B,D*) were performed in cooperation with Dr. Gerrit Wolters-Eisfeld in the group of Prof. Melitta Schachner.

The 70 kDa L1 Fragment Originates at the Plasma Membrane upon Serine Protease Activity and is Transported to the Nucleus via the Late Endosomal Compartment

Generation and nuclear import of a transmembrane NCAM fragment can be inhibited by the serine protease inhibitor aprotinin (Kleene et al., 2010), showing that a serine protease is involved in its generation. It has been shown that this transmembrane NCAM fragment is transported from the plasma membrane to the nucleus via the ER and the cytoplasm (Kleene et al., 2010). To analyze whether the generation and nuclear translocation of the 70 kDa L1 fragment also depends on serine protease-mediated proteolytic processing, cultured cerebellar neurons were treated with antibody 557 in the presence of the serine protease inhibitor aprotinin. The soluble nuclear fractions and the culture supernatants showed reduced levels of the 70 kDa and 135 kDa fragments, respectively, when aprotinin was present during neuronal stimulation with L1 antibody 557 (Fig. 3A), compared to levels in the absence of aprotinin. This result affirms that generation of both L1 fragments depends on a serine protease activity.

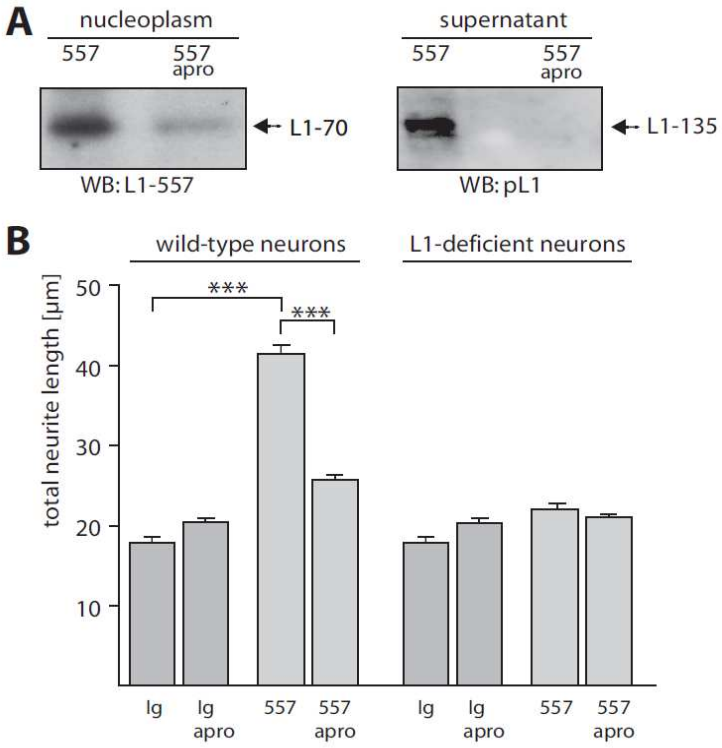

FIGURE 3. **Generation, nuclear import of the transmembrane 70 kDa L1 fragment and L1-induced neurite outgrowth depend on serine protease activity.** Cerebellar neurons from wild-type (*A,B*) or L1-deficient mice (*B*) were treated with the L1 antibody 557 or a non-immune rat antibody (Ig) in the absence or presence of aprotinin (apro). *A*, nuclear fractions containing nucleoplasm and the cell culture supernatant were subjected to Western blot analysis using antibody 557 (L1-557) or polyclonal L1 antibody (pL1) against the entire extracellular domains. Identical amounts of nucleoplasmic proteins were loaded. Loading was controlled by probing the Western blot (WB) with HP1γ antibody (not shown). Different L1 fragments are indicated. Representative blots from one of three independent experiments with similar results are shown. *B*, cerebellar neurons were incubated with antibody 557 or a non-immune antibody (Ig) in the absence or presence of aprotinin (apro). Total lengths of neurites were measured, and mean values ± S.E. from three independent experiments are shown (***, $p < 0.001$).

To connect the serine protease-dependent generation of the 70 and 135 kDa fragments with L1 mediated functions, such as neurite outgrowth, the effect of aprotinin on antibody 557-triggered neuritogenesis was investigated. Neurite outgrowth was promoted in the presence of antibody 557 relative to that determined in the presence of a control antibody, while the concomitant presence of aprotinin resulted in a reduction of this antibody-triggered neurite

outgrowth to a value comparable to that observed in the presence of the control antibody (Fig. 3B). Neither aprotinin nor antibody 557 affected neurite outgrowth of L1-deficient neurons (Fig. 3B). This result shows that L1-induced neurite outgrowth depends on the serine protease-mediated cleavage of L1 and suggests that the generation of the 70 and 135 kDa fragments and the nuclear import of the 70 kDa fragment play a role in L1-triggered neurite outgrowth.

To unravel the pathway of the 70 kDa fragment from the cytosol towards the nucleus, subcellular fractions were analyzed by Western blot for the presence of this fragment after cell surface biotinylation. SH-SY5Y cells highly expressing L1 were chosen for this experiment, as they can be cultured in large quantities in contrast to cerebellar neurons. Upon stimulation of SH-SY5Y cells with function-triggering polyclonal L1 antibody, the 70 kDa fragment was seen in non-nuclear and nuclear fractions (Fig. 4A), while it was not detectable after treatment with the non-immune antibody (Fig. 4A). These results show that L1 stimulation of SH-SY5Y cells leads to the generation and nuclear import of the 70 kDa fragment similarly to cerebellar neurons. After cell surface biotinylation and treatment with antibody 557 or control antibody, SH-SY5Y cells were subjected to subcellular fractionation to isolate plasma membrane (PM), smooth ER (SER), rough ER (RER), cytoplasmic (cyt), endosomal and nuclear fractions. To track the pathway of the biotinylated 70 kDa L1 fragment after its generation at the plasma membrane, biotinylated proteins were isolated from these fractions using streptavidin beads and subjected to Western blot analysis using antibody 557. Upon treatment of cells with this antibody, the 70 kDa fragment as well as the 200 kDa full-length L1 and L1 fragments of 90, 140 and 180 kDa were detected as biotinylated proteins in a plasma membrane-enriched fraction, whereas only biotinylated

140, 180 and 200 kDa bands, but not the biotinylated 70 and 90 kDa fragments were detectable in this fraction from cells treated with a non-immune control antibody (Fig. 4B). The biotinylated 70 kDa transmembrane L1 fragment was detected in the cytoplasmic fraction (Fig. 4C), RER- and SER-enriched fraction (Fig. 4D) after antibody stimulation of SH-SY5Y cells. This fragment was not detectable when cells were incubated with the control antibody (Fig. 4C,D). The 90, 140, 180 and 200 kDa L1 bands seen in the plasma membrane fraction were not found in the cytoplasmic fraction (Fig. 4C) enriched in the cytoplasmic marker protein actin, but devoid of the ER marker protein PDI and the nuclear marker protein HP1γ (Fig. 4E). ER fractions (Fig. 4D) which were enriched in PDI, but devoid of actin and HP1γ (Fig. 4E) also lacked these bands. Analysis of fractions obtained after sucrose gradient centrifugation of microsomes showed biotinylated 140, 180 and 200 kDa L1 bands in gradient fractions enriched in Alix and Tsg101, two marker proteins of late endosomes and/or multivesicular bodies (MVB), when cells were treated with L1 or control antibody (Fig. 4F). However, biotinylated 70 and 90 kDa fragments were only seen in those endosomal fractions when cells were incubated with L1 antibody, but were not detectable in fractions from cells treated with control antibody (Fig. 4F). Moreover, only the biotinylated 70 kDa fragment was detectable in the nuclear fractions containing soluble or chromatin-associated proteins after stimulation of cells with L1 antibody, but not after application of control antibody (Fig. 4G).

Since the 70 kDa fragment was present in a MVB enriched fraction and since exosomes which mediate the transport of proteins from one cell to another are formed in MVB (Lakkaraju and Rodriguez-Boulan, 2008; Simons and Raposo, 2009), the presence of biotinylated 70 kDa L1 fragment in exosomes was analyzed. The biotinylated 70 kDa fragment was detectable in exosomes

isolated from the cell-free culture supernatant of cells treated with L1 antibody, but not in the supernatant of cells treated with control antibody (Fig. 4H). In summary, these results indicate that L1 stimulation leads to generation of the 70 kDa fragment at the plasma membrane and that this fragment is translocated from the plasma membrane to the ER, late endosomal compartment and/or MVB, cytoplasm and nucleus.

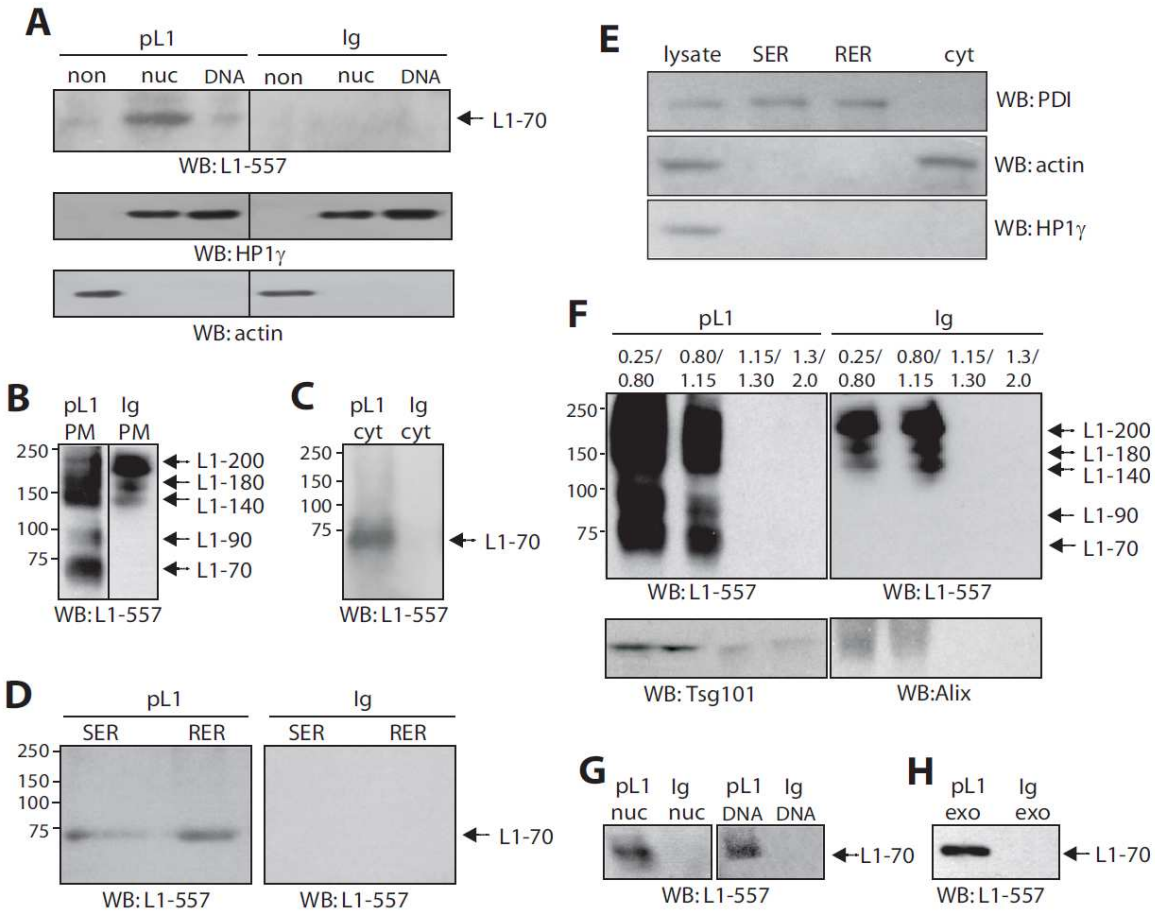

FIGURE 4. **Intracellular path of the 70 kDa fragment.** *A*, non-nuclear (non) and nuclear fractions containing nucleoplasmic (nuc) or chromatin-associated (DNA) proteins were isolated from SH-SY5Y cells treated with polyclonal L1 antibody (pL1) or control antibody (Ig). Identical amounts of protein were loaded, and the HP1γ and actin antibodies were used in Western blot (WB) analysis to control loading of non-nuclear or nuclear proteins. *B–H*, SH-SY5Y cells were subjected to cell surface biotinylation followed by incubation with polyclonal L1 antibody (pL1) or non-immune control antibody (Ig) and subcellular fractionation. Using streptavidin-coupled beads, biotinylated proteins were isolated from plasma membrane (PM) (*B*), cytoplasmic (cyt) (*C,E*), SER and RER (*D,E*), and endosomal (*F*) fractions, from nuclear fractions containing nucleoplasmic (nuc) or chromatin-associated (DNA) proteins (*G*), or from exosomes (exo) (*H*). Biotinylated L1-immunoreactive bands

were detected by Western blot analysis using antibody 557 (L1-557). SER, RER, and cytoplasmic fractions were probed by Western blot analysis with actin, HP1γ, and protein-disulfide isomerase (PDI) antibodies (*E*), and gradient fractions were probed by Western blot analysis with Tsg101 and Alix antibodies (*F*). The different L1 fragments are indicated. Lanes not adjacent to each other but from the same blot are indicated by dividing lines. Representative blots from three (*A*) or two (*B–H*) independent experiments with similar results are shown. *B–G*, the protein concentrations in the fractions were determined, and identical amounts of protein from the corresponding fraction were loaded. The experiments (*A*,*B*,*C*,*D*,*E*,*F*) were performed in cooperation with Dr. Gerrit Wolters-Eisfeld and Dr. Gunjan Joshi in the group of Prof. Melitta Schachner.

Release of the 70 kDa L1 Fragment from Endosomal Membranes into the Cytoplasm Depends on ESCRT-III-associated Proteins

To explore if the 70 kDa fragment is released from the ER and/or the late endosomal compartment into the cytoplasm, ER and endosomal fractions containing this biotinylated fragment were isolated from SH-SY5Y cells after cell surface biotinylation and stimulation with polyclonal L1 antibody. Fractions were incubated at 37°C or 4°C with cytoplasm prepared from untreated SH-SY5Y cells or with buffer used for the isolation of the cytoplasmic fraction. After incubation, ultracentrifugation and isolation of biotinylated proteins from the resulting pellet and supernatant fractions, Western blot analysis with antibody 557 was carried out. Biotinylated 70 kDa L1 fragment was observed only in the pellet fraction, but not in the supernatant fraction when ER fractions were used for incubation (data not shown), indicating that this fragment is not released from the ER membranes.

After incubation of the endosomal fractions with the cytoplasmic fraction at 37°C, the biotinylated 70 kDa fragment was no longer detectable – neither in the pellet nor in the supernatant fraction (data not shown) – suggesting that it was rapidly proteolytically degraded at this temperature. However, the biotinylated 70 kDa fragment was detectable in the pellet and supernatant

fraction after incubation of the endosomal fraction with buffer or the cytoplasmic fraction at 4°C (Fig. 5A). Interestingly, the amounts of biotinylated 70 kDa fragment released from the endosomes into the supernatant when the endosomal fraction was incubated with buffer were higher than the amounts seen when the fraction was incubated with cytoplasm (Fig. 5A). In summary, cytoplasmic components are not essential for the release of the 70 kDa fragment from endosomal membranes. Hence, this fragment was speculated to be released from the membrane through endosome-associated proteins.

ESCRT proteins (endosomal sorting complex required for transport) and ESCRT-associated proteins adhere to endosomal membranes, mediate the formation of MVB, and play crucial roles in endosomal protein sorting and intra-endosomal trafficking (Slagsvold et al. 2006; van der Goot and Gruenberg, 2006; Tanaka et al., 2008). Thus, their potential involvement in release of the 70 kDa fragment from endosomal membranes into the cytoplasm was assessed by testing whether antibodies against distinct ESCRT proteins and ESCRT-associated proteins could affect the release of the 70 kDa fragment. Endosomal fractions isolated from SH-SY5Y cells positive for the biotinylated 70 kDa fragment upon cell surface biotinylation and antibody stimulation were incubated with buffer in the absence or presence of antibodies against the ESCRT-I subunit Tsg101, against ESCRT-III-associated Vps4 (key player in membrane abscission reactions during endosomal sorting) and antibodies against the ESCRT-III-associated proteins Alix and CHMP1. After incubation and ultracentrifugation, the biotinylated proteins were isolated from the supernatant fractions and subjected to Western blot analysis with L1 antibody to elucidate how the release of the biotinylated 70 kDa fragment was affected in the presence of these antibodies. The amount of biotinylated fragment

released into the supernatant was reduced in the presence of Vps4, Alix or CHMP1 antibodies when compared to the level obtained in the absence of antibodies or in the presence of Tsg101 antibody which did not block the release of the 70 kDa fragment from the endosomes (Fig. 5B). These findings indicate that Vps4, CHMP1 or Alix antibodies inhibit the release of the 70 kDa fragment from the endosomal membrane. To further substantiate this notion, endosomal fractions containing the 70 kDa fragment were prepared from mouse brains and incubated at 4°C in the absence or presence of Vps4, CHMP1, Alix and Tsg101 antibodies. After ultracentrifugation and immunoprecipitation of L1 from the supernatant fractions, Western blot analysis of the immunoprecipitates revealed that the release of the 70 kDa fragment from endosomes was drastically reduced in the presence of Vps4, CHMP1 or Alix antibodies relative to the level observed in the absence of antibodies or in the presence of Tsg101 antibody (Fig. 5C). The combined results suggest that blocking Vps4, CHMP1 or Alix by antibodies impairs release of the 70 kDa fragment and, thus, provide evidence that these ESCRT-III-associated proteins are involved in the release of this fragment from the endosomal membrane into the cytoplasm.

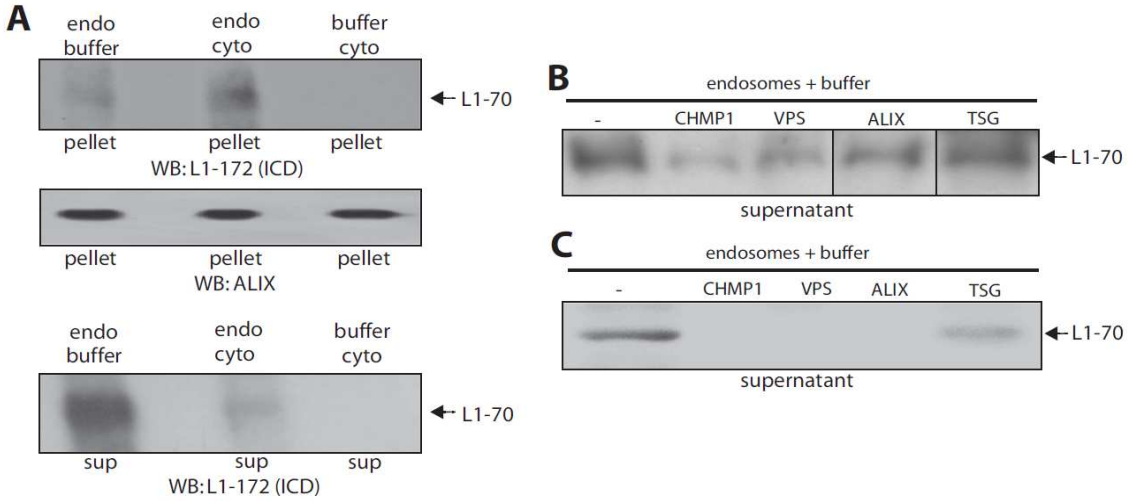

FIGURE 5. **Release of the 70 kDa L1 fragment from endosomal membranes into cytoplasm depends on ESCRT-associated proteins.** *A,B*, SH-SY5Y cells were subjected to cell surface biotinylation followed by incubation with polyclonal L1 antibody and isolation

of endosomal fractions (endo) containing the biotinylated L1 fragment. This fraction was incubated with a cytoplasmic fraction (cyto) isolated from untreated cells or cells treated with the buffer used for isolation of the fractions in the absence (*A,B*) or presence of antibodies against CHMP1, Vps4 (VPS), Alix (ALIX), and Tsg101 (TSG) (*B*). After ultracentrifugation, biotinylated proteins were isolated from the resulting pellet (*A*) and supernatant (sup) (*A,B*), and the biotinylated 70 kDa fragment was detected in the pellet and supernatant fractions by Western blot (WB) analysis using L1 antibody 172-R. *C*, endosomal fractions containing the 70 kDa fragment were isolated from mouse brain homogenate and incubated at 4°C in the absence or presence of antibody against CHMP1, Vps4, Alix, or Tsg101. After ultracentrifugation, the resulting supernatants were used for immunoprecipitation with antibody 172-R, and immunoprecipitates were subjected to Western blot analysis using antibody 172-R. *A*, Alix antibody was used in Western blot analysis of the pellet fraction to control that similar amounts of endosomes were used in the experiment and were reisolated after incubation. *A,B*, lanes not adjacent to each other but from the same blot are indicated by dividing lines. The experiments were performed two times with identical results. The experiments (*A,B*) were performed in cooperation with Dr. Gunjan Joshi in the group of Prof. Melitta Schachner.

Nuclear Import of the 70 kDa Fragment Depends on Importin and Is Accompanied by Import of CHMP1

To demonstrate the nuclear import of the 70 kDa fragment from the cytoplasm after its release from the endosomal membrane, an *in vitro* nuclear import assay was performed. Nuclei from untreated SH-SY5Y cells and a cytoplasmic fraction from SH-SY5Y cells after cell surface biotinylation and L1 stimulation were isolated. The cytoplasm containing the biotinylated 70 kDa fragment or the buffer which had been used for the isolation of the cytoplasm was incubated with the nuclei from untreated cells. Biotinylated proteins which had been translocated from the cytoplasmic fraction into the nucleus were then isolated from the nuclei and subjected to Western blot analysis with antibody 172-R. The biotinylated 70 kDa fragment was hardly detectable after incubation with buffer, but detectable after incubation with cytoplasm containing this fragment (Fig. 6A). In a similar experiment, nuclei from mouse brains were incubated with a cytoplasmic fraction isolated from brain homogenate or with buffer used for fractionation. After re-isolation of the nuclei, Western blot analysis with antibody 172-R showed that the 70 kDa

fragment was imported into the nuclei after incubation with cytoplasm, but not after incubation with buffer alone (Fig. 6B).

As nuclear import of proteins depends on nuclear localization signals, to which importins bind (Chook and Süel, 2011; Marfori et al., 2011) and since L1 contains two putative monopartite nuclear localization signals (K_{1147}RSK and K_{1235}KEK), it was interesting to see whether the nuclear import of the 70 kDa fragment is importin-dependent. To test this, cytoplasm obtained from SH-SY5Y cells after L1 stimulation and containing the 70 kDa fragment was incubated with nuclei from non-stimulated cells in the absence and presence of antibodies against importin-α or -β to block the nuclear import. After isolating the nuclei, nuclear extracts were subjected to Western blot analysis using the antibody 172-R. In the absence of antibodies, the 70 kDa fragment was detected after incubation with cytoplasm containing the fragment. The fragment was not detectable after incubation with cytoplasm in the presence of the importin antibodies (Fig. 6D). These results demonstrate that the nuclear import of the 70 kDa fragment depends on importin-α and -β.

Since L1 is released from exosomes in a CHMP1-dependent manner and given that CHMP1 recruits the polycomb protein BMI to the subnuclear regions of condensed chromatin (Stauffer et al., 2001), the potential of CHMP1 to act as a nuclear carrier protein involved in the nuclear import of the 70 kDa L1 fragment was tested. Particularly, the association of the cytoplasmatic 70 kDa fragment with CHMP1 and, in parallel, importin-β and importin-α in SH-SY5Y cells was investigated. Western blot analysis after immunoprecipitation showed that both importins and CHMP1 were co-immunoprecipitated with L1 from the cytoplasmic fraction of SH-SY5Y cells (Fig. 6C).

To explore if CHMP1 plays a role in the nuclear import of the 70 kDa fragment, nuclei from mouse brains were incubated with a post-nuclear supernatant isolated from mouse brains or with buffer in the absence and presence of CHMP1 antibody or, for control, in the presence of Vps4, Alix or non-immune antibodies. Nuclear extracts were then prepared and subjected to Western blot analysis with antibody 172-R. The 70 kDa fragment was present in the nuclear extract after incubation of nuclei with the post-nuclear supernatant in the absence of antibodies and in the presence of the non-immune, Vps4 and Alix antibodies, while it was not detectable in untreated nuclei and in nuclei after incubation with buffer or with post-nuclear supernatant in the presence of the CHMP1 antibody (Fig. 6E). These results indicate that the import of the 70 kDa fragment from the cytoplasm into the nucleus is mediated by importin-α/β and CHMP1-dependent mechanisms.

To investigate whether L1 proteolytic processing as well as the nuclear import of the 70 kDa fragment affects nuclear import of CHMP1, cerebellar neurons were treated with antibody 557 or non-immune antibodies and subcellular fractions were isolated and subjected to Western blot analysis with CHMP1 antibody. Relative to the CHMP1 levels observed upon treatment with the non-immune antibody, the level of membrane-associated CHMP1 was strongly decreased, while the level of DNA-associated CHMP1 was strongly increased upon stimulation with L1 antibody (Fig. 6F). The level of cytoplasmic CHMP1 was not significantly altered (Fig. 6F). This result indicates that the nuclear import of the 70 kDa fragment is accompanied by an altered subcellular localization and nuclear import of CHMP1.

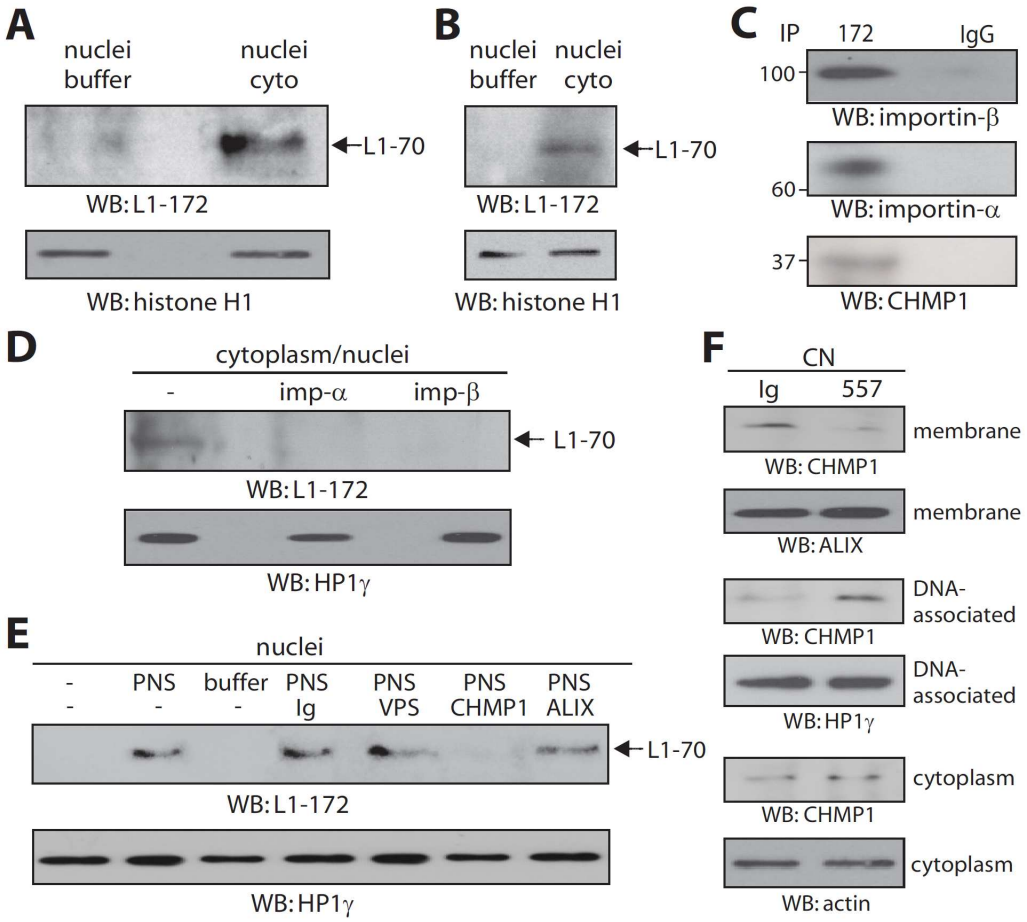

FIGURE 6. **Nuclear import of the 70 kDa L1 fragment depends on importins and CHMP1.** *A,D*, SH-SY5Y cells were subjected to cell surface biotinylation followed by incubation with polyclonal L1 antibody and isolation of a cytoplasmic fraction containing biotinylated L1 fragment. *A*, this fraction or the buffer that was used for the isolation of the fractions was incubated with nuclei isolated from untreated cells. After re-isolation of nuclei by centrifugation, biotinylated proteins were isolated from nuclei, and the biotinylated L1 fragment was detected by Western blot (WB) analysis using L1 antibody 172-R (L1-172). *B*, cytoplasmic fraction (cyto) and nuclei were isolated from mouse brain, and nuclei were incubated either with the cytoplasmic fraction or with the buffer used for the isolation of the cytoplasmic fraction. After re-isolation, nuclei were subjected to Western blot analysis using antibody 172-R. *C*, the cytoplasmic fraction isolated from L1-stimulated SH-SY5Y cells was subjected to immunoprecipitation (IP) using antibody 172-R (172) or non-immune mouse control antibody (IgG), and immunoprecipitates were probed by Western blot analysis using antibodies against importin-α (imp-α), importin-β (imp-β), and CHMP1. *D*, cytoplasmic fraction from L1-stimulated SH-SY5Y cells containing the 70 kDa fragment was incubated with nuclei of untreated SH-SY5Y cells in the absence or presence of importin-α (imp-α) or importin-β antibody (imp-β). After re-isolation of nuclei by centrifugation, nuclear extract was subjected to Western blot analysis using antibody 172-R (L1-172). *E*, nuclei from mouse brain were incubated either with a post-nuclear supernatant (PNS) isolated from mouse brain homogenate and containing the 70 kDa fragment or with the buffer used for isolation of the post-nuclear supernatant in the absence or presence of Vps4 (VPS), CHMP1, and Alix (ALIX) antibodies or non-immune control antibody (Ig).

After re-isolation of the nuclei, nuclear extract was subjected to Western blot analysis using antibody 172-R (L1-172). *F*, cerebellar neurons (CN) were treated with antibody 557 or non-immune antibody (Ig) and subjected to subcellular fractionation. Fractions enriched in membranes and DNA-associated and cytoplasmic proteins were subjected to Western blot analysis using CHMP1 antibody. *A,B,D,E*, histone H1 or HP1γ antibody was used in Western blot analysis of the pellet fraction to control that similar amounts of nuclei were used in the experiment and were re-isolated after incubation. F, Alix, HP1γ, and actin antibodies were used in Western blot analysis to control loading of membrane, DNA-associated, or cytoplasmic proteins. A–F, the experiments were performed three times with identical results, and representative blots are shown. The experiments in (*C,D*) were performed in cooperation with Ute Bork in the group of Prof. Melitta Schachner.

Generation of the 70 kDa Fragment Requires Sumoylation of L1 at Lysine$_{1172}$ (K_{1172}), and Its Nuclear Import is Mediated by the Nuclear Localization Signal K_{1147}RSK

Analysis of the intracellular trafficking of the 70 kDa fragment in SH-SY5Y cells after cell surface biotinylation and L1 stimulation showed that the fragment is internalized after its generation at the plasma membrane and transported via a late endosomal compartment to the nucleus. Initially, it was analyzed if the 70 kDa L1 fragment is ubiquitinated, since this post-translational modification mediates endocytosis of transmembrane proteins (Kirkin and Dikic, 2007; Clague and Urbé, 2010). Sumoylation of the fragment was tested in addition, since the intracellular domain of L1 contains two potential sites for the attachment of small ubiquitin-like modifier (sumo) proteins and since sumoylation plays an important role in nucleocytoplasmic transport (Geiss-Friedlander and Melchior, 2007).

The SUMOsp 2.0 software (Ren et al., 2009) identified the non-canonical type II sumoylation site $GK_{1235}KE$ and the type I sumoylation site $MK_{1172}DE$ which represents a canonical ΨKXE sumoylation motif (Ψ is A, I, L, M, P, F or V; X can be any amino acid) in the intracellular domain of L1. Only the

full-length L1 appeared to be ubiquinated, as no further ubiquitinated L1 fragments were observed in immunoprecipitates from mouse brain homogenate with L1 or poly-ubiquitin antibody and Western blot analysis with poly-ubiquitin or L1 antibody, respectively (Fig. 7A). The pan-sumo antibody detected a 70 kDa band in the L1 immunoprecipitates of plasma membrane-enriched mouse brain fraction (Fig. 7B). When these immunoprecipitates were probed with antibody 557 for Western blot analysis, not only the 70 kDa but also the 200 kDa full-length L1, the 140 and 180 kDa L1 fragments were detectable (Fig. 7B). In L1 immunoprecipitates from endosomal mouse brain fractions, the 70 kDa fragment was detected with pan-sumo and L1 antibodies. The 140, 180 and 200 kDa L1 bands were detected by L1, but not by pan-sumo antibodies (Fig. 7C). After L1 immunoprecipitation from SER or cytoplasmic mouse brain fractions, only the 70 kDa fragment was detected by Western blot analysis with pan-sumo antibody (Fig. 7D) and antibody 557 (data not shown). When the non-immune antibody was used for immunoprecipitation, no sumo- or L1-immunoreactive bands were observed in either immunoprecipitate (Fig. 7B-D).

In addition, it was analyzed if the nuclear and endosomal 70 kDa fragments observed after L1-stimulation of SH-SY5Y cells were also sumoylated. SH-SY5Y cells were therefore treated with the polyclonal L1 antibody or with the non-immune antibody. Nuclear fractions and exosomes were then isolated and subjected to immunoprecipitation with L1 or non-immune antibodies. The pan-sumo antibody detected the 70 kDa fragment in L1 immunoprecipitates from exosomes (Fig. 7E) and from the nuclear fraction upon L1 stimulation (Fig. 7F). This fragment did not appear when the non-immune antibody was used for immunoprecipitation or treatment of the cells

(Fig. 7F). The combined results suggest that the sumoylated 70 kDa fragment is present at the plasma membrane and in endosomes, ER, cytoplasm, exosomes and nuclei.

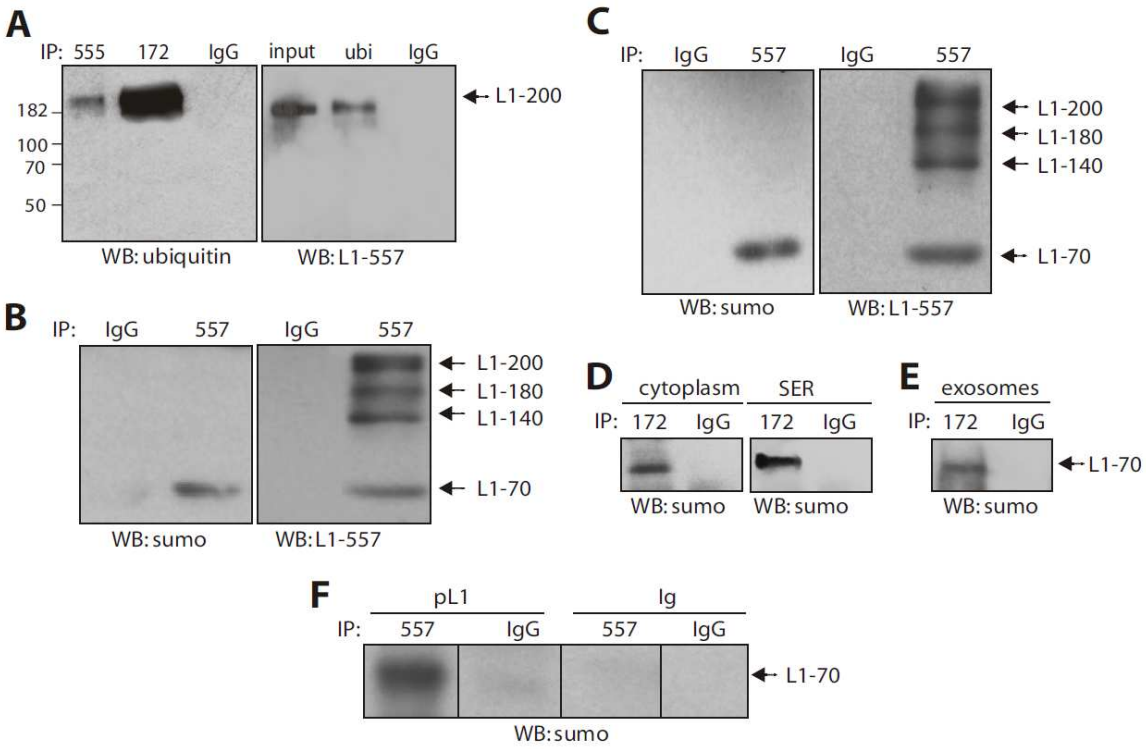

FIGURE 7. **The 70 kDa L1 fragment which is sumoylated, but not ubiquitinated, is generated at the plasma membrane by a serine protease.** *A*, mouse brain homogenate was subjected to immunoprecipitation using antibodies 555 or 172-R (172), ubiquitin antibody (ubi), or non-immune control antibody (IgG). Homogenate (input) and immunoprecipitates were subjected to Western blot (WB) analysis with ubiquitin antibody or antibody 557 (L1-557). *B–D*, fractions enriched in plasma membranes (*B*), endosomes (*C*), or cytoplasm or SER (*D*) were isolated from mouse brain and subjected to immunoprecipitation using antibody 557 or non-immune control antibody (IgG) followed by Western blot analysis with antibody 557 (L1-557) (*B,C*) or with a pan-SUMO antibody (sumo) (*B–D*). *E,F*, SH-SY5Y cells were treated with polyclonal L1 antibody (pL1) (*E,F*) or control antibody (Ig) (*F*), and exosomes isolated from the cell-free cell culture supernatant (*E*) or nuclear protein extracts (*F*) were subjected to immunoprecipitation (IP) using antibody 557 or non-immune control antibody (IgG) (*E,F*). Immunoprecipitates were probed by Western blot analysis with a pan-SUMO antibody. *A–E*, the full-length L1 and the different L1 fragments are indicated by arrows. Lanes not adjacent to each other but from the same blot are indicated by dividing lines. The experiments were performed three times with identical results, and representative blots are shown. The experiments (*A,B,C*) were performed in cooperation with Dr. Gerrit Wolters-Eisfeld in the group of Prof. Melitta Schachner.

To test if the sumoylation and nuclear localization signals are required for the nuclear import of the 70 kDa fragment, the potential sumoylation site at K_{1172}, the nuclear localization signal at K_{1147}, as well as the potential sumoylation site and nuclear localization signal at K_{1235} were disrupted by site directed mutagenesis (Fig. 8A). HEK293 cells, which do not express L1, were either mock-transfected or transfected with an empty expression vector or with expression vectors encoding full-length non-mutated or mutated L1. After stimulation of these cells with polyclonal L1 antibody, non-nuclear and nuclear fractions were isolated. In parallel, cell surface biotinylation was carried out before L1 stimulation and biotinylated proteins were isolated from the cell lysates.

Biotinylated full length L1 was detected in the non-nuclear fraction and on the cell surface after transfection with non-mutated full-length L1, while no L1 positive band was seen in non-transfected or mock-transfected cells (Fig. 8B, C). Moreover, the levels of mutated L1 in non-nuclear fractions and at the cell surface were higher than the levels of wild-type non-mutated L1 (Fig. 8B,C). This would suggest that the L1 mutants with disruption of the putative sumoylation site at K_{1172} are shedded to a lesser extent from the plasma membrane upon L1 stimulation. L1 immunoprecipitates from non-nuclear fractions of cells expressing wild-type L1, L1 with a mutated sumoylation site or nuclear localization signal at K_{1235} (Fig. 8D) were positive for the 70 kDa fragment. This fragment was not detected in the L1 immunoprecipitates of cells expressing the mutation of the sumoylation site at K_{1172}, and strongly reduced in the fraction from cells expressing L1 with mutated nuclear localization signal at K_{1147} (Fig. 8D). Correspondingly, the 70 kDa fragment was abundant in nuclear fractions containing chromatin-associated proteins from cells expressing wild-type L1 or the mutation of the sumoylation site and

the nuclear localization signal at K_{1235}, whereas no 70 kDa fragment was found in the nuclear fraction isolated from cells expressing the mutation of the nuclear localization signal at K_{1147} or the sumoylation site at K_{1172} (Fig. 8E). These observations indicate that sumoylation of L1 at K_{1172} is a prerequisite for the extracellular proteolytic cleavage of L1 and the generation of the 70 kDa fragment. The mutation of the nuclear localization signal at K_{1147} has a minor effect on the generation of the 70 kDa fragment; its main effect is to impair the nuclear import of this fragment. The motif at K_{1235} does not affect sumoylation and, thus, generation and nuclear import of the 70 kDa fragment.

To verify that the 70 kDa fragment is sumoylated at K_{1172}, HEK293 cells were co-transfected with full length L1 and GFP-tagged sumo-1, sumo-2 or sumo-3. A GFP-positive band of 100 kDa was observed in cell lysates obtained upon L1 stimulation and co-transfection of L1 with the GFP-tagged sumo isoforms, but not when cells were mock-transfected or transfected with L1 alone (Fig. 8F). GFP has a molecular weight of approximately 30 kDa, and attachment of one GFP-sumo to L1 resulted in appearance of a 100 kDa L1 band. There was no evidence of additional higher molecular weight bands at 130 kDa as would be expected for additional sumoylation events. Thus, these data suggest that the 100 kDa L1 fragment comprises one GFP (30 kDa), one sumo-protein (~ 15 kDa) and a 55 kDa L1 remnant. Hence, the 70 kDa L1 fragment remains to be a monosumoylated 55 kDa L1 stump.

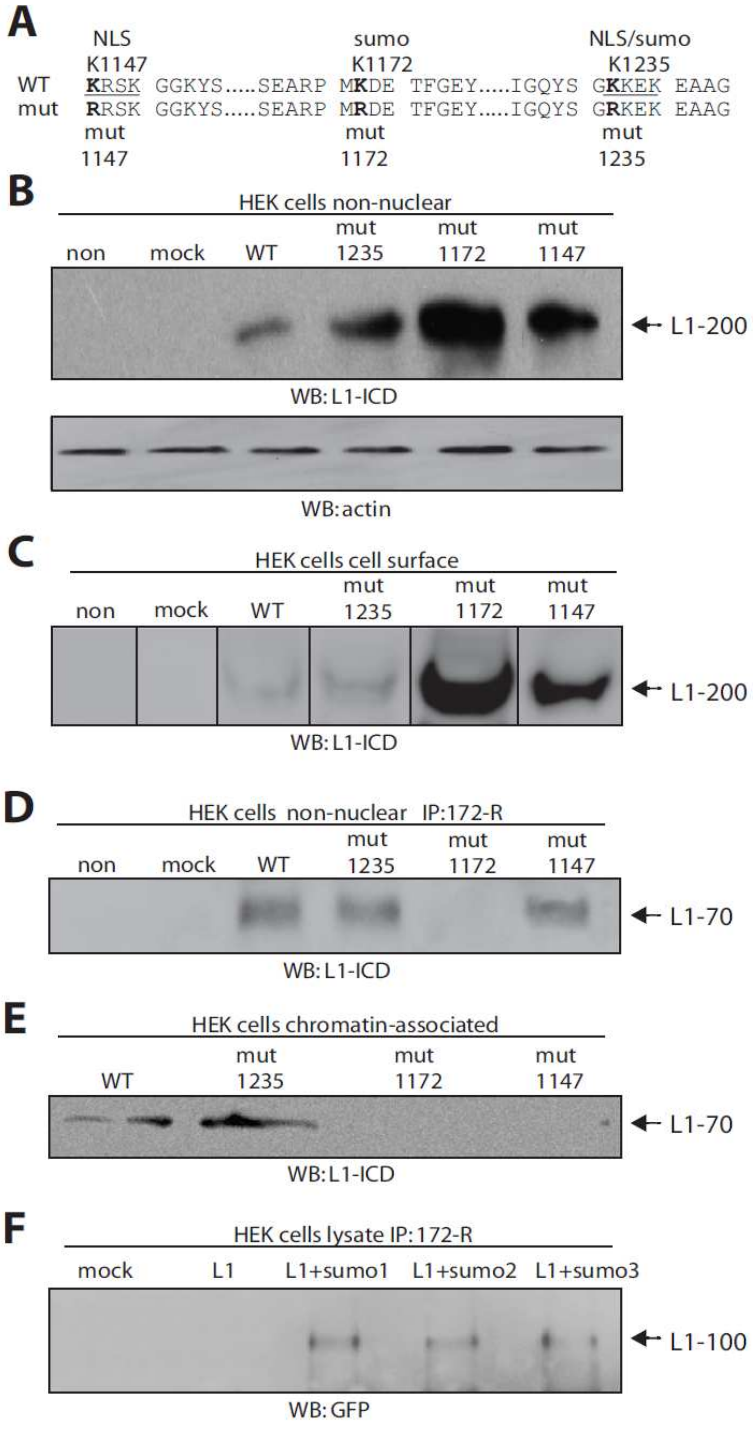

FIGURE 8. **Sumoylation of L1 at Lysine$_{1172}$ is required for generation of the 70 kDa L1 fragment, and the nuclear import of this fragments depends on the nuclear localization signal Lysine$_{1147}$.** *A*, schematic presentation of the nuclear localization signals (NLS) and potential sumoylation sites (sumo) in the intracellular domain of mouse L1 at Lys$_{1147}$, Lys$_{1172}$, and Lys$_{1235}$, respectively. Sequences of wild-type L1 (WT) and L1 mutants (mut) are shown. *B–F*, untransfected (non) (*B–D*) or mock-transfected HEK cells (*B–D,F*), HEK cells transiently transfected with wild-type (WT) or mutated L1 (*B–E*), and HEK cells transfected with L1 alone or co-transfected with L1 and GFP-tagged sumo-1, sumo-2, or

sumo-3 (*F*) were incubated with polyclonal L1 antibody. *C*, non-transfected and transfected cells were subjected to cell surface biotinylation before treatment with L1 antibody. Biotinylated proteins (*C*), non-nuclear fractions (*B,D*) and nuclear fractions containing chromatin-associated proteins (*E*) were isolated from lysates of transfected cells. Non-nuclear fractions (D), nuclear fractions containing chromatin-associated proteins (E) and cell lysates (F) were subjected to immunoprecipitation using antibody 172-R. *B–F*, Western blot (WB) analysis using antibody 172-R (*B–E*) or using a GFP antibody (*F*) is shown. Full-length L1 and the different L1 fragments are indicated by arrows. Lanes not adjacent to each other but from the same blot are indicated by dividing lines. *B*, actin antibody was used in Western blot analysis to control loading. *B–F*, the experiments were performed two times with identical results and representative blots are shown.

Generation of the 70 kDa Fragment Correlates with Brain Development, Regeneration after Spinal Cord Injury and Degeneration in an Alzheimer´s Disease Mouse Model

Measurements of fragment levels in brain homogenates from mice of different ages were performed to deduce functional roles of the 70 kDa L1 fragment *in vivo*. Western blot analysis of brain homogenates using antibody 172-R showed that the levels of full-length L1 (200 kDa) and of a double band at ~70 kDa increased from embryonic day 14 until birth (Fig. 9). These enhanced levels remained constant until postnatal day 3 (P3), but declined thereafter (Fig. 9). Since antibody 172-R does not discriminate between the transmembrane 70 kDa fragment identified in this study and the previously described transmembrane 80 kDa fragment resulting from the cleavage within the third FNIII domain (Kalus et al., 2003), antibody 555 recognizing the 70 kDa, but not the 80 kDa fragment, was used for Western blot analysis to specifically determine the level of the 70 kDa fragment during development. This antibody detected the full-length L1 and the 70 kDa fragment. Levels of full-length L1 were increased from embryonic day 14 to postnatal day 4 and then declined, while significant levels of the 70 kDa fragment were detectable from postnatal days 0 to 5 (Fig. 9). These results indicate that the 70 kDa fragment is mainly generated during early postnatal development and suggest

that its occurrence may underlie or accompany cellular events that take place between birth and postnatal day 5, such as neuronal migration and differentiation, neuritogenesis, and developmental synaptogenesis.

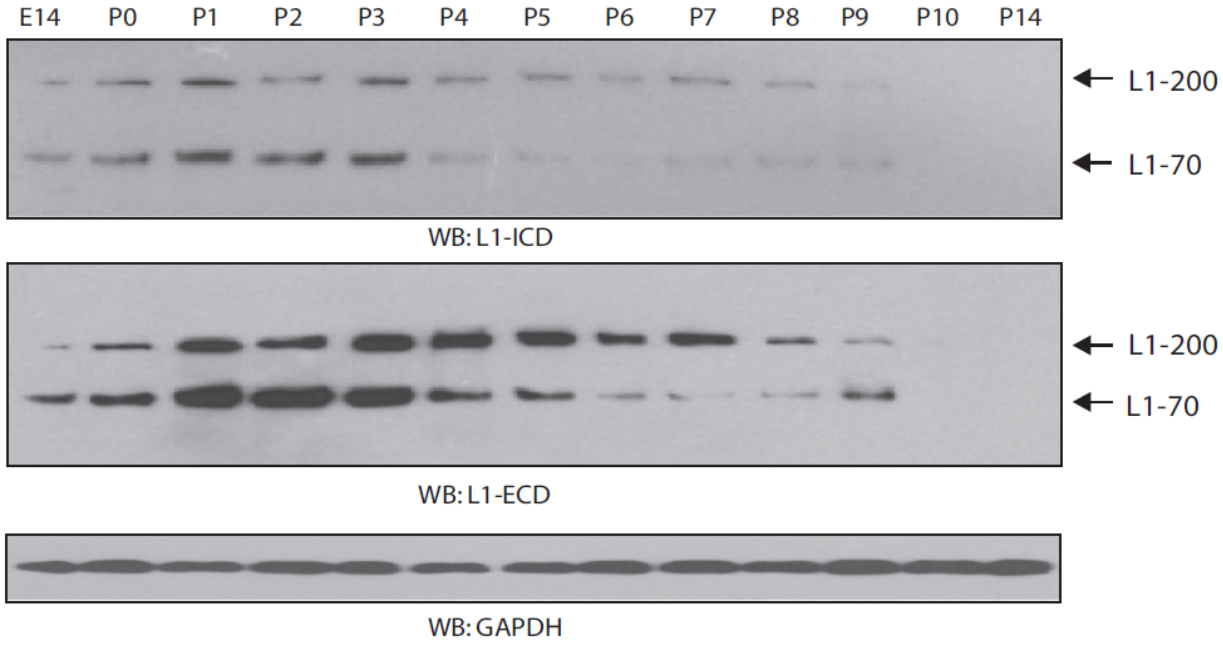

FIGURE 9. **Levels of the 70 kDa fragment are increased during early postnatal development.** Brain homogenates from mice of different ages were subjected to Western blot (WB) analysis with the antibodies 172-R (L1-ICD; upper panel) or 555 (L1-ECD; lower panel). Representative results from one of four animals per group are shown after different exposure times of the blots. GAPDH antibody was used in Western blot analysis to control loading. The experiments were performed two times with identical results, and representative blots are shown.

In addition, levels of this fragment were also determined in spinal cord segments rostral to the lesion site one week after spinal cord injury of adult mice. In particular, expression of L1 in the thoracic spinal cord was monitored, since this is the segment where the severed axons of the corticospinal tract regrow and strongly express L1 following spinal cord injury (Jakeman et al., 2006). Western blot analysis of thoracic spinal cord segments from non-injured mice and mice at 7th day after injury using antibody 172-R showed increased levels of the 70 kDa L1 fragment in the injured spinal cord relative to the low levels in spinal cords from non-injured mice. The level of the 80 kDa fragment

was reduced (Fig. 10A). L1 antibody 555 recognizing the 70 kDa fragment and the soluble extracellular 140 kDa fragment, but not the transmembrane 80 kDa fragment, detected enhanced levels of the 70 kDa fragment and reduced levels of the 140 kDa fragment after spinal cord injury relative to the levels observed in non-injured animals. The level of full-length L1 detected by both antibodies was not significantly altered after injury when compared to non-injured mice (Fig. 10A). These results led to the assumption that the expression of full-length L1 is not changed by injury, whereas altered proteolytic processing leads to a decrease in the level of the transmembrane 80 kDa fragment in favor of the generation of the transmembrane 70 kDa fragment.

To determine whether the 70 kDa fragment is altered in the degenerating brain, a mouse model of Alzheimer's disease was used. APPPS1-21 mice develop amyloid plaques in the cortex and hippocampus at three months of age, due to overexpression of a mutated form of amyloid precursor protein (Swedish mutation) and increased γ-secretase activity (Radde et al., 2006).

Homogenates from the frontal cortex of APPPS1-21 showed markedly reduced levels of the 70 kDa fragment and slight reduction in the amount of the full-length L1 and the 80 kDa fragment compared to those in homogenates from wild-type mice on Western blot analysis using antibody 172-R (Fig. 10B). In addition, highly increased levels of the γ-secretase cleavage product with an apparent molecular weight of 28 kDa (Riedle et al., 2009) were observed in APPPS1-21 mice (Fig. 10B). These findings relate the higher γ-secretase activity in APPPS1-21 mice to an increased cleavage of the 70 kDa fragment and a negligible cleavage of full-length L1 and the 80 kDa fragment. Moreover, the generation of the 28 kDa fragment resulting from the

predominant cleavage of the 70 kDa fragment suggests possible roles of L1's processing in the pathogenesis of Alzheimer's disease.

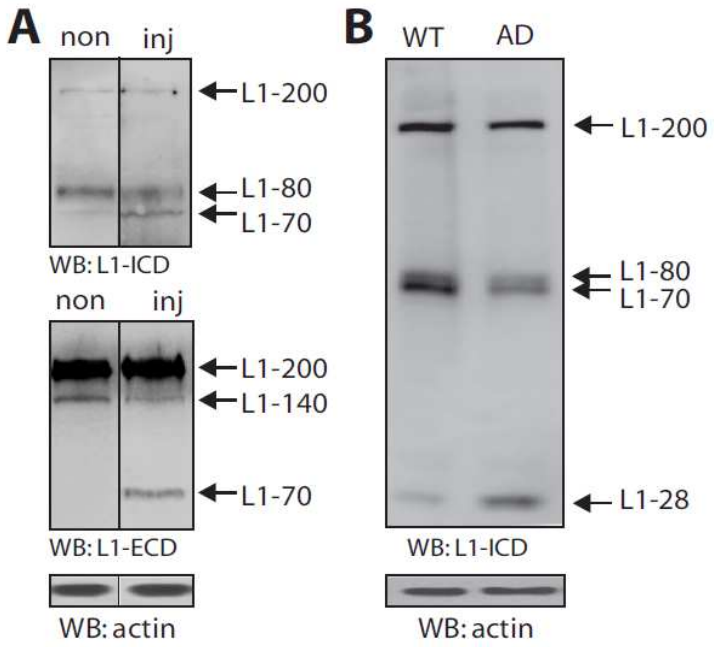

FIGURE 10. **Levels of the 70 kDa fragment are increased after spinal cord injury, but decreased in a mouse model of Alzheimer's disease.** *A,* 1 week after spinal cord injury, segments of the thoracic spinal cord rostral to the lesion site were taken from injured mice (inj), and corresponding segments were taken from non-injured mice (non). The segments were subjected to Western blot analysis with antibodies 172-R (L1-ICD; upper panel) and 555 (L1-ECD; lower panels). *B*, homogenates from the frontal cortex of wild-type (WT) and APPPS1–21 (AD) mice were subjected to Western blot analysis using antibody 172-R. *A,B*, actin antibody was used in Western blot analysis to control loading. The experiments were performed two times with identical results, and representative blots are shown. The experiments (*A,B*) were performed in cooperation with Nevena Djogo and Dr. Igor Jakovcevski in the group of Prof. Melitta Schachner.

A 30 kDa Intracellular L1 Fragment Is Generated and Translocated into the Nucleus upon Antibody-mediated L1 Stimulation

Western blot analysis using the monoclonal L1 antibody 172-R against an intracellular epitope showed that stimulation of cerebellar neurons with L1 antibody 557 can also trigger the generation of a smaller L1 fragment. This

fragment with an apparent molecular mass of 30 kDa was observed in the non-nuclear and nuclear fractions containing either soluble or chromatin-associated nuclear proteins (Fig. 11A). The 30 kDa fragment was not detected in fractions from neurons which were mock-treated or treated with a non-immune control antibody (Fig. 11A) or when L1-deficient cerebellar neurons were stimulated with antibody 557 (Fig. 11B). In addition, the 30 kDa fragment was not detectable by antibodies directed against extracellular epitopes (Fig. 11C), suggesting that this L1 fragment lacks the extracellular entity and comprises only the intracellular domain.

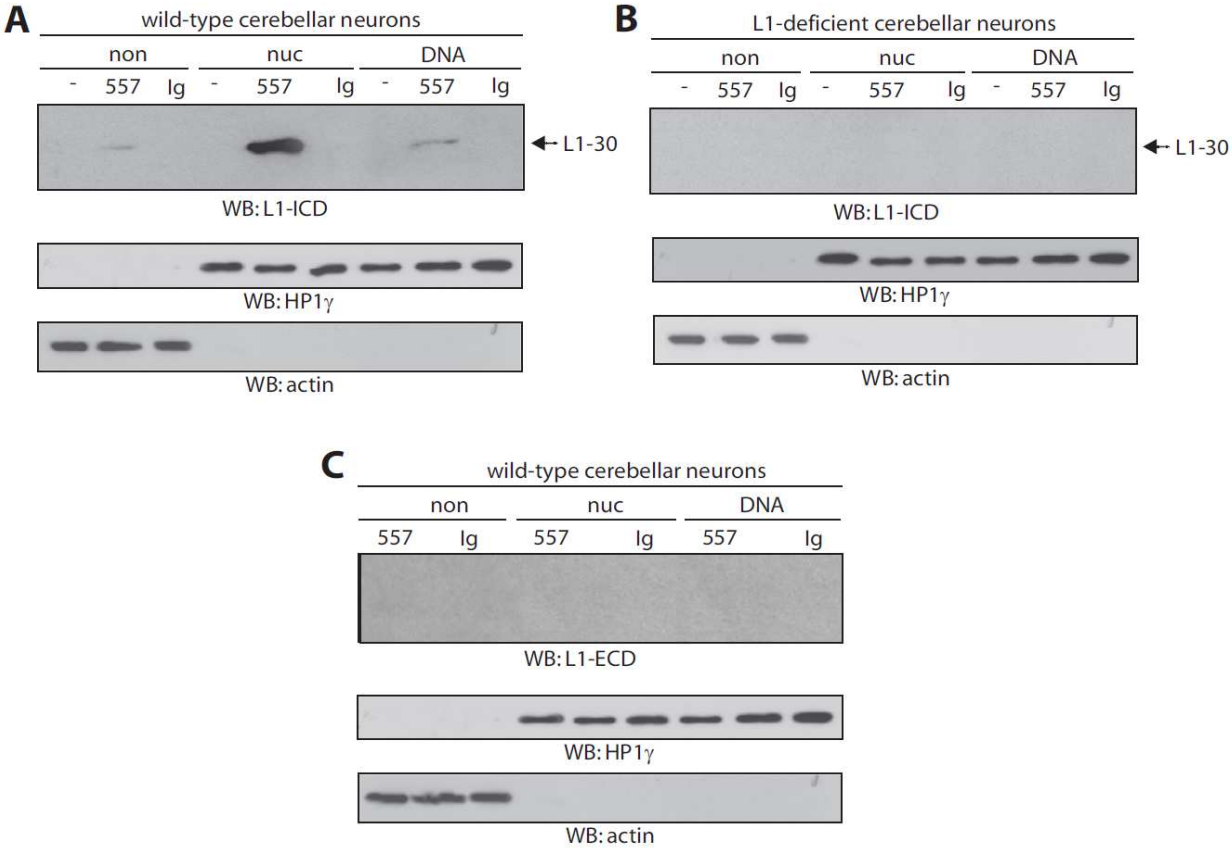

FIGURE 11. **Stimulation of L1 signaling leads to generation and nuclear import of a 30 kDa L1 fragment.** Wild-type (*A,C*) and L1-deficient (*B*) cerebellar neurons were mock-treated or treated with the L1 antibody 557 or a non-immune rat control antibody (Ig) (*A-C*). Non-nuclear fractions (non), nuclear fractions containing nucleoplasm (nuc) or chromatin-associated proteins (DNA) were isolated and subjected to Western blot analysis using the antibody 172-R directed against the intracellular domain of L1 (L1-ICD) (*A,B*) or using the antibody 557 against an extracellular L1 epitope (L1-ECD) (*C*). Experiments were repeated four times with similar results. Respresentative Western blots of the 30 kDa L1 fragment are chosen. Controls for purity of fractions are shown. The experiment in (*A*) was

performed in cooperation with Dr. Gerrit Wolters-Eisfeld in the group of Prof. Melitta Schachner.

Generation and Nuclear Import of the 30 kDa L1 Fragment Depend on Sumoylation of L1 at Lysine$_{1172}$ (K_{1172}) and on the Nuclear Localization Signal K_{1147}RSK

As shown in Fig. 7, the L1-induced generation of the 70 kDa L1 fragment depends on sumoylation of the canonical motif MK_{1172}DE. In addition, nuclear import of this fragment is mediated by the monopartite nuclear localization signal K_{1147}RSK. Neither the putative non-canonical sumoylation site GK_{1235}KE nor the putative nuclear localization signals K_{1235}KEK are involved in generation or nuclear import of the 70 kDa fragment.

Since the 30 kDa fragment of L1 comprises both motifs MK_{1172}DE and GK_{1235}KE, it was examined if this fragment is also sumoylated. For this, immunoprecipitation was performed using a nuclear extract from adult mouse brain and a pan-sumo antibody followed by Western blot analysis with L1 antibody 172-R against an intracellular epitope. A 30 kDa fragment was detectable in immunoprecipitates obtained with the pan-sumo antibody (Fig. 12A), but not in immunoprecipitates obtained with a non-immune control antibody (Fig. 12A). It was then interesting to see, if the sumoylation motif MK_{1172}DE or GK_{1235}KE (Fig. 12B) and/or the nuclear localization signal K_{1147}RSK or K_{1235}KEK (Fig. 12B) are also essential for the generation and/or nuclear import of the 30 kDa fragment. To answer this question, L1-deficient HEK293 cells were mock-transfected or transfected with an empty expression vector or with expression vectors encoding the full-length wild-type neuronal isoform of L1 or neuronal L1 with either a mutation of K_{1172}, K_{1147} or K_{1235} (Fig. 12B). After stimulation of the cells with antibody 557, subcellular

fractionation and immunoprecipitation with antibody 172-R, the 30 kDa fragment was observed in L1 immunoprecipitates isolated from the non-nuclear fraction of cells expressing wild-type L1 or the L1 mutants with disrupted sumoylation site and nuclear localization signal at K_{1235} (Fig. 12C). The fragment was not seen in L1 immunoprecipitates from cells expressing the mutation of the sumoylation site at K_{1172} (Fig. 12C). The fragment level was reduced in the fractions from cells with mutated nuclear localization signal at K_{1147} (Fig. 12C). The 30 kDa fragment was detected in nuclear fractions containing chromatin-associated proteins from cells expressing wild-type L1 or the mutation of the sumoylation site and of the nuclear localization signal at K_{1235} (Fig. 12D). No 30 kDa fragment was detectable in the nuclear fraction isolated from cells expressing the mutated nuclear localization signal at K_{1147} or the mutated sumoylation site at K_{1172} (Fig. 12D). These results indicate that sumoylation of L1 at K_{1172} is required for generation of the 30 kDa fragment, that the nuclear localization signal at K_{1147} mediates the nuclear import of the fragment, and that the motif at K_{1235} does not play a role in generation or nuclear import of the 30 kDa fragment.

To demonstrate the sumoylation at K_{1172} of the 30 kDa fragment, HEK293 cells were stimulated with antibody 557 after co-transfection of full-length L1 and GFP-tagged sumo-1, sumo-2 or sumo-3. GFP-positive bands of 60 kDa were observed in cell lysates obtained upon co-transfection of L1 with GFP-tagged sumo-2 and -3 and L1 stimulation (Fig. 12E). However, no band was detectable in lysates isolated from L1-stimulated mock-transfected cells, cells transfected only with L1, and cells co-transfected with L1 and GFP-tagged sumo-1 (Fig. 12E). As similarly described for the 70 kDa L1 fragment, attachment of one sumo tagged with GFP with an estimated molecular mass of approximately 45 kDa induces the appearance of a 60 kDa L1 band.

Regarding the molecular weight of sumo und GFP and subtracting it from the molecular weight of the 60 kDa L1 band, it becomes clear that the 30 kDa L1 fragment represents a monosumoylated 15 kDa L1 remnant.

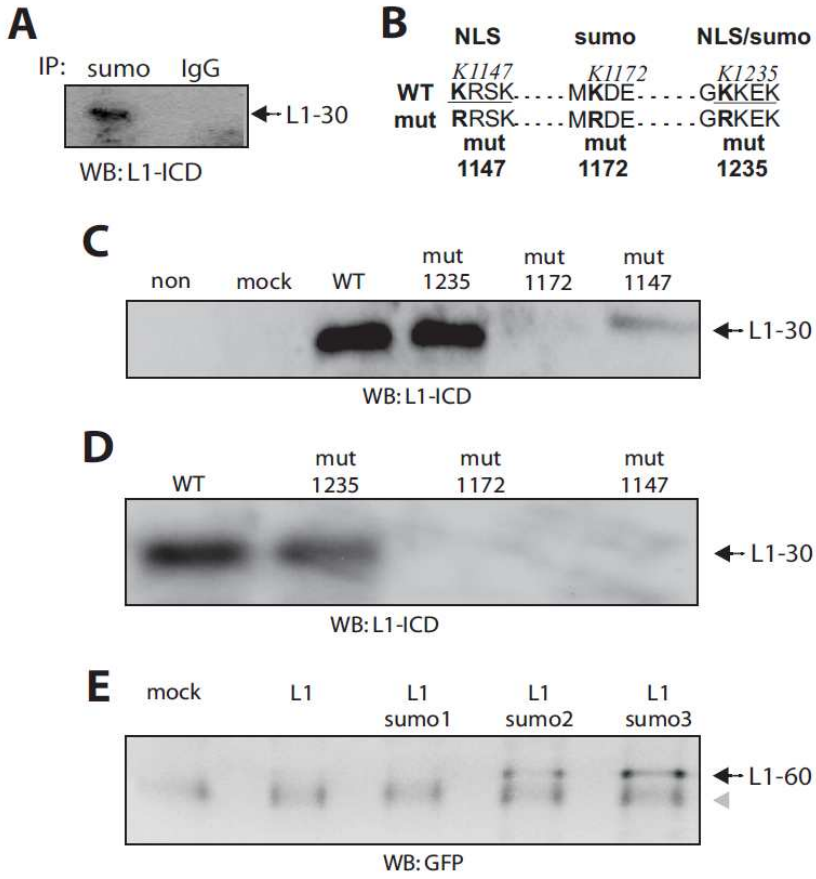

FIGURE 12. **Generation and nuclear import of the 30 kDa L1 fragment depend on sumoylation at K_{1172} and on the nuclear localization signal at K_{1147}.** *A*, nuclear extracts isolated from mouse brains were subjected to immunoprecipitation using a pan-sumo antibody (sumo) or a non-immune control antibody (IgG). Immunoprecipitates were probed by Western blot analysis with L1 antibody 172-R. *B*, schematic presentation of the nuclear localization signals (NLS) and potential sumoylation sites (sumo) in the intracellular domain of mouse L1 at K_{1147}, K_{1172} and K_{1235}, respectively. Sequences of the wild-type L1 (WT) and L1 mutants (mut) are shown. Non-transfected (non) or mock-transfected HEK cells (*C,E*), HEK cells transiently transfected with wild-type (WT) or mutated L1 (*C,D*) and HEK cells transfected with L1 alone or co-transfected with L1 and GFP-tagged sumo-1, sumo-2 or sumo-3 (*E*) were stimulated with a polyclonal L1 antibody. Non-nuclear fractions (*C*) and nuclear fractions containing chromatin-associated proteins (*D*) were isolated from lysates of the transfected cells. Non-nuclear fractions (*C*) or cell lysates (*E*) were subjected to immunoprecipitation using antibody 172-R. Western blots with L1 antibody 172-R (*C,D*) or GFP antibody (*E*) are shown. Different L1 fragments are indicated by arrows and an unspecific band is indicated by an arrowhead (*E*). Experiments were performed twice with similar results.

Simultaneous Generation of the 30 and 55 kDa L1 Fragments Depends on Serine and Aspartyl Protease but not on Metalloprotease or Presenilin/γ-secretase Activities

The apparent molecular mass of the intracellular 30 kDa fragment is similar to the previously described intracellular 28 kDa fragment which is generated by presenilin/γ-secretase mediated cleavage of the 32 kDa fragment deriving from the cleavage of L1 by metalloproteases (Maretzky et al., 2005; Riedle et al., 2009). Thus, it was essential to clarify whether the generation and nuclear import of the 30 kDa fragment depend on the proteolytic processing by presenilin/γ-secretase and/or metalloproteases. Since a serine protease was proposed to mediate L1 antibody-triggered generation of the transmembrane 70 kDa L1 fragment, it was of interest to determine whether a serine protease might also be involved in the generation of the 30 kDa fragment.

To evaluate whether presenilin/γ-secretase, metalloproteases and/or serine proteases generate the 30 kDa fragment, cerebellar neurons were treated with antibody 557 in the absence or presence of the presenilin/γ-secretase inhibitor DAPT, the metalloprotease inhibitor GM6001 or the serine protease inhibitor aprotinin. The levels of the 30 kDa fragment were then determined in nucleoplasmic fractions by Western blot analysis with antibody 172-R. The 30 kDa fragment levels were increased in antibody 557-stimulated neurons in the presence of DAPT when compared to the levels seen with stimulation in the absence of inhibitors, and were not altered upon L1 stimulation in the presence of GM6001 (Fig. 13A). The 30 kDa fragment was neither detectable after application of the antibody 557 in the presence of aprotinin, nor after treatment of neurons with non-immune antibody (Fig. 13A). This result

shows that the 30 kDa fragment does not originate from metalloprotease- or presenilin/γ-secretase-mediated cleavages and, thus, differs from the previously described intracellular 28 kDa fragment generated by the presenilin/γ-secretase-mediated cleavage of the transmembrane 32 kDa fragment that emerges from a membrane-proximal cleavage by metalloprotease activities (Riedle et al., 2009). Moreover, generation of the 30 kDa fragment is accompanied by simultaneous generation of a corresponding 55 kDa fragment. It was therefore necessary to unravel the trafficking pathway of this corresponding fragment within the cell.

Interestingly, a 55 kDa L1 fragment was observed in exosomes isolated from cell culture supernatants after cell surface biotinylation and L1 stimulation of neuroblastoma cells when probed with antibody 557 directed against an extracellular L1 epitope. Furthermore, only the 70 kDa fragment, but not the 55 kDa fragment, was seen with the antibody 172-R which is directed against an intracellular L1 epitope (Fig. 13B) and no L1 fragment was detectable by either antibody when cells were treated with a non-immune antibody (Fig. 13B). These observations raised the question of whether the 55 kDa fragment is also generated in the brain *in vivo* by proteolytic processing at the plasma membrane and/or in endosomal compartments where exosomes are formed.

In a first step, generation of the 55 kDa fragment was triggered *in vitro* by incubating freshly prepared homogenates from adult mouse brain at 37°C. Full-length L1, but not the 55 kDa fragment, could be detected by Western blot analysis with antibodies 172-R or 557 after incubating the homogenate at 4°C (Fig. 13C). Upon incubation at 37°C, the 55 kDa fragment was detected, and a concomitant reduction of the full-length L1 level was observed (Fig. 13C). Next, homogenates were incubated at 37°C in the absence or presence

of protease inhibitors. Subsequently, fractions enriched in plasma membranes or endosomes were isolated and the level of the 55 kDa fragment in these fractions was determined. In the absence of inhibitors, low levels of the 55 kDa fragment were seen in the plasma membrane-enriched fraction (Fig. 13D) and in the endosomal fraction enriched in Tsg101 (Fig. 13E), a marker protein of multivesicular bodies (MVBs) in which exosomes are formed (Lakkaraju and Rodriguez-Boulan, 2008; Simons and Raposo, 2009). In comparison to the levels observed in the absence of inhibitors, the 55 kDa fragment level in the plasma membrane-enriched fraction was increased in the presence of DAPT (presenilin/γ-secretase inhibitor), while the level of the full-length L1 was reduced (Fig. 13D). The levels of the 55 kDa fragment were reduced in the presence of the serine protease inhibitor aprotinin or the proteasome inhibitor MG132, which has broad substrate specificity and inhibits cysteine, serine and aspartyl proteases (Fig. 13D). The level of full-length L1 was not changed in the presence of aprotinin or MG132 (Fig. 13D). An enhanced level of the 55 kDa fragment in the endosomal/MVBs fraction was also observed in the presence of DAPT relative to the level observed in the absence of inhibitor, while reduced levels of the 55 kDa fragment were observed in the presence of aprotinin or MG132 (Fig. 13E). The level of Tsg101 was not altered in the presence of either inhibitor (Fig. 13E). These results suggest that the membrane-bound 55 kDa fragment is generated at the plasma membrane as a result of a serine and an aspartyl and/or a cysteine protease activity. The low levels of the membrane-bound 55 kDa indicate that most of this fragment is degraded after its internalization, and that a small portion of the 55 kDa fragment is translocated to late endosomes/MVBs from where it could be shuttled to exosomes.

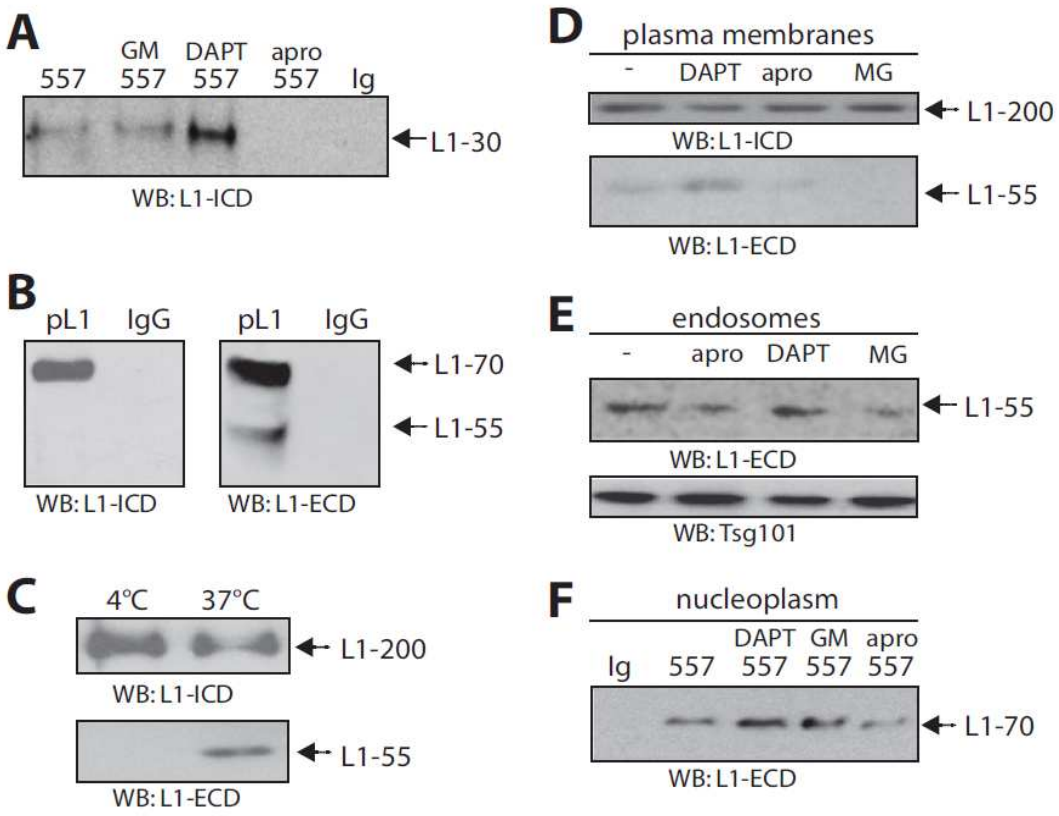

FIGURE 13. **Generation of the 30 kDa intracellular and corresponding membrane-bound 55 kDa fragment depends on an aspartyl protease activity.** *A*, cerebellar neurons were treated with the L1 antibody 557 or a non-immune rat antibody (Ig) in the absence or presence of DAPT, GM6001 (GM) or aprotinin (apro). Nuclear fractions containing nucleoplasm were probed by Western blot analysis with the antibody 172-R (L1-ICD). *B*, cerebellar neurons were incubated with L1 antibody 557 or a non-immune rat antibody (Ig). Exosomes were isolated from the cell culture supernatants and subjected to Western blot analysis with antibodies 557 (L1-ECD) or 172-R (L1-ICD). *C*, mouse brain homogenates were incubated at 4°C or 37°C and subjected to Western blot analysis with antibodies 172-R (L1-ICD) or 557 (L1-ECD). *D,E*, after incubating mouse brain homogenate at 37°C in the absence or presence of MG132 (MG), DAPT or aprotinin (apro), fractions enriched in plasma membranes (*D*) or endosomes (*E*) were isolated and subjected to Western blot analysis with antibodies 172-R (L1-ICD) (D) or 557 (L1-ECD) (*D,E*) or with a Tsg101 antibody (*E*). *F*, cerebellar neurons were treated with the antibody 557 or a non-immune rat antibody (Ig) in the absence or presence of DAPT, GM6001 (GM) or aprotinin (apro). Nuclear fraction containing nucleoplasm was isolated and probed by Western blot analysis using antibody 557 (L1-ECD). *A-F*, full-length L1 and different L1 fragments are indicated. Experiments were repeated three times with similar results. The experiments (*A,D,E,F*) were performed in cooperation with Dr. Gunjan Joshi in the group of Prof. Melitta Schachner.

The 30 and 55 kDa Fragments Derive from the Aspartyl Protease-mediated Cleavage of the Transmembrane 70 kDa Fragment

The 30 and 55 kDa fragments are not generated by a presenilin/γ-secretase or metalloprotease activity, but by a protease that is inhibited by aprotinin. Aprotinin inhibited generation of the 70 kDa fragment (see Fig. 2). Since the three fragments are not generated in the presence of aprotinin, it was conceivable that the 30 and 55 kDa fragments derive from the 70 kDa fragment. To test this idea, it was first determined whether the generation and nuclear import of the 70 kDa fragment does not require presenilin/γ-secretase or metalloprotease activities. The level of the 70 kDa fragment in a nuclear fraction containing soluble nuclear proteins was determined upon L1-stimulation of cerebellar neurons in the absence or presence of the presenilin/γ-secretase inhibitor DAPT or the metalloprotease inhibitor GM6001. In parallel, treatment of cerebellar neurons with antibody 557 was carried out in the presence of the serine protease inhibitor aprotinin. In the presence of DAPT the 70 kDa fragment level in the nuclear fraction was enhanced, whereas it remained unaltered by GM6001 and was decreased in the presence of aprotinin (Fig. 13F). The fragment was not detectable upon treatment of neurons with non-immune control antibody (Fig. 13F). This result indicates that the 70 kDa fragment is not generated by presenilin/γ-secretase, but rather is a substrate for this enzyme complex: inhibition of presenilin/γ-secretase by DAPT increases the level of the 70 kDa fragment. As the increase or decrease of the 70 kDa fragment amounts by DAPT or aprotinin are accompanied by corresponding increase or decrease in levels of the 30 and 55 kDa fragments, these fragments were thought to be cleavage products of the transmembrane 70 kDa fragment.

The generation of the 55 kDa fragment was reduced in the presence of the cysteine and aspartyl protease inhibitor MG132. Based on this observation, the generation of the 70 kDa and/or 30 kDa fragment was investigated in L1-stimulated cerebellar neurons in the absence and presence of the aspartyl protease inhibitor pepstatin or the cysteine protease inhibitor leupeptin. In parallel, neurons were treated with aprotinin. Compared to the levels seen in the absence of inhibitors, elevated levels of the 70 kDa fragment were observed in the presence of pepstatin, but not in the presence of leupeptin (Fig. 14A). The 30 kDa fragment was observed in the absence of inhibitors or in the presence of leupeptin, but it was not detectable in the presence of pepstatin (Fig. 14A). Neither the 30 kDa nor the 70 kDa fragment were seen in the presence of aprotinin (Fig. 14A). These results provide evidence that cleavage of the 70 kDa fragment by an aspartyl protease results in generation of the 30 kDa fragment.

The 30 kDa L1 Fragment Regulates L1-induced Neuronal Migration but not Neuritogenesis

Based on the observations before, it was investigated whether the L1-induced generation and nuclear import of the 30 kDa fragment plays a role in regulating L1-induced functions, considering not only neurite outgrowth, but also neuronal migration (Lindner et al., 1983). Therefore, the effect of pepstatin and aprotinin on antibody 557-triggered neurite outgrowth and neuronal migration in cultures of dissociated cerebellar neurons and/or cerebellar explants was analyzed. As expected, the total length of neurites extending from dissociated neurons or from explants was increased by antibody 557 treatment in comparison to the control (Fig. 14B-D). This enhanced neurite outgrowth was not affected by pepstatin, whereas aprotinin completely abolished the L1-promoted neurite outgrowth (Fig. 14B-D).

Neuronal migration out of the explant core was accelerated by antibody 557 when compared to migration observed in the presence of a non-immune control antibody. This L1-stimulated enhancement of neuronal migration was reduced to control values by both pepstatin and aprotinin (Fig. 14B-D). These results show that L1-induced neuronal migration depends on the aspartyl and serine protease-mediated cleavage of L1 and suggest that the 30 kDa fragment generated from the 70 kDa fragment, which is involved in neuritogenesis, plays a role in L1-triggered neuronal migration.

Cathepsin E Activity Generates the Intracellular 30 kDa L1 Fragment

Given that the aspartyl proteases cathepsin E and D are inhibited by pepstatin, they appear to be prominent candidates for the generation of the 30 kDa L1 fragment and regulation of neuronal migration. Cathepsin E and D are highly homologous but differ in their subcellular localization. Cathepsin D is predominantly found in lysosomes, while cathepsin E has been found in endosomes, endoplasmic reticulum, Golgi apparatus and at the plasma membrane (Zaidi et al., 2008). Moreover, cathepsin E has been detected at the cytoplasmic surface of the plasma membrane (Ueno et al., 1989) and is evenly distributed within the cytoplasm (Saku et al., 1990; Hara et al., 1993). Considering this and the finding that the 30 and 55 kDa fragments are generated by an aspartyl protease at the plasma membrane, it was conceivable that cathepsin E could be the protease cleaving the 70 kDa fragment to 30 and 55 kDa fragments. To approach this, the impact of reduced cathepsin E expression on the generation of the 30 kDa fragment in cerebellar neurons transfected with cathepsin E siRNA was investigated.

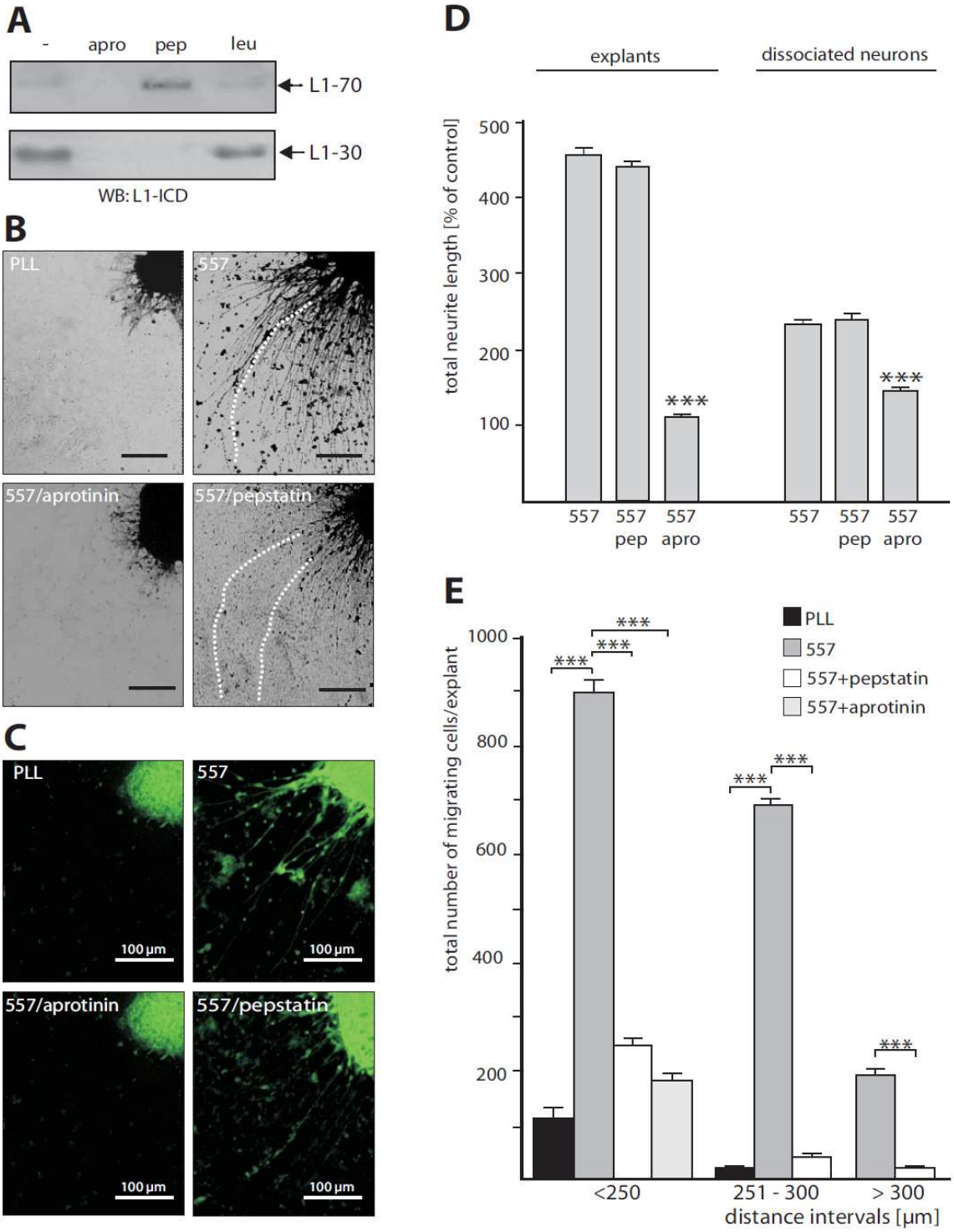

FIGURE 14. **L1-triggered neuronal migration depends on generation of the 30 kDa L1 fragment upon aspartyl protease activity.** *A*, cerebellar neurons were treated with antibody 557 in the absence or presence of pepstatin (pep), leupeptin (leu) or aprotinin (apro). Cell lysates were probed by Western blot analysis with antibody 172-R (L1-ICD). *B-E*, dissociated cerebellar neurons (*D*) or cerebellar explants (*B-E*) were maintained on substrate-coated antibody 557 or rat non-immune control antibody in the absence or presence of aprotinin or pepstatin. *B,C,* phase contrast images (*B*) or βIII-tubulin

immunostainings (*C*) of representative explants are shown. Bars represent 100 μm and dashed lines indicate representative neurites extending from the microexplants. *D*, neurite outgrowth was determined by measuring the total lengths of neurites. Neurite lengths relative to those observed on PLL (as control set to 100%) are shown. *E*, numbers of cell bodies within different distance intervals are shown (*** $p<0.005$, oneway-ANOVA with Tukey's Multiple Comparison Test).

As controls, cathepsin D or control siRNAs were used. Upon transfection with cathepsin E or D siRNA, the levels of cathepsin E and D proteins, respectively, were reduced (Fig. 15A). When the transfected neurons were treated with non-immune control antibody, no 30 kDa and 70 kDa fragments were observed by Western blot analysis of cell lysates with antibody 172-R (Fig. 15A). Both fragments were detected in cell lysates of L1-stimulated neurons transfected with cathepsin D and control siRNA, while only the 70 kDa fragment, but not the 30 kDa fragment was present in the cell lysate of L1-stimulated neurons upon transfection with cathepsin E siRNA (Fig. 15A). This result shows that the 30 kDa fragment is generated from the 70 kDa fragment by cathepsin E.

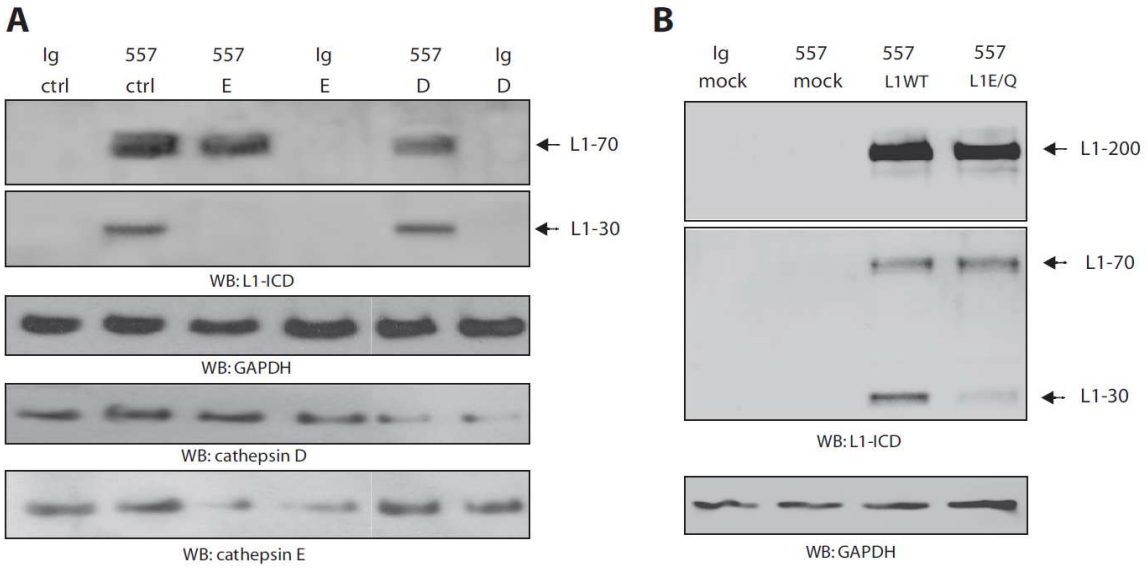

FIGURE 15. **L1-triggered generation of the 30 kDa L1 fragment depends on cathepsin E.** *A*, cerebellar neurons were transfected with control (ctrl), cathepsin E (E), or cathepsin D (D) siRNA and thereafter treated with L1 antibody 557 or a non-immune rat control

antibody (Ig). Cell lysates were probed by Western blot analysis using antibody 172-R (L1-ICD) or antibodies against cathepsin E or D as well as GAPDH to control for loading. *B*, mock-transfected HEK cells or HEK cells transfected with wild-type (L1WT) or mutated L1 (L1E/Q) were incubated with antibody 557 or a non-immune rat control antibody (Ig). Cell lysates were subjected to Western blot analysis using antibody 172-R (L1-ICD) and GAPDH antibody to control loading. The full-length L1 (L1-200) and the different L1 fragments are indicated by arrows. Experiments were repeated three times with similar results.

Cathepsin E Cleaves the Intracellular Domain of L1 at E_{1167}A to Generate the 30 kDa Fragment

Cathepsin E, which is active even at neutral pH shows a rather selective cleavage of a Glu-Ala (EA) bond within the B chain of insulin at pH 7.4 (Athauda et al., 1991). Since an EA sequence is present two amino acids proximal to the sumoylation site MKDE in the intracellular domains of all known mammalian L1 family molecules and of the chicken and pufferfish L1 homologs, we hypothesized that cathepsin E cleaves the sumoylated 70 kDa fragment at this site to generate the sumoylated 30 kDa fragment. To test this assumption, L1-deficient HEK293 cells were mock-transfected or transfected with expression vectors encoding full-length wild-type L1 or mutated L1 bearing an exchange of glutamic acid (E) to glutamine (Q) at position 1167 and treated with antibody 557 or non-immune control antibody. Western blot analysis of the cell lysates showed that wild-type and mutant full-length 200 kDa L1 molecules were equally well expressed, while no L1 was detectable in the lysates of mock-transfected cells (Fig. 15B). The 30 and 70 kDa fragments were seen in lysates of cells expressing wild-type L1 (Fig. 15B). In lysates of cells expressing mutant L1, the 70 kDa fragment level was slightly increased, whereas levels of the 30 kDa fragment were strongly reduced (Fig. 15B). This result indicates that mutation of the potential cathepsin E cleavage site within the intracellular domain of L1 prevents the generation of the 30 kDa fragment and suggests that cathepsin E-mediated cleavage of the sumoylated 70 kDa

fragment at the E_{1167}A bond next to the sumoylation site at K_{1172} within the intracellular domain produces the 30 kDa fragment.

To further substantiate the dependency of L1-induced cell migration on cathepsin E cleavage at E_{1167}, a scratch assay was performed with HEK cells that had been transfected either with L1, L1 mutated at E_{1167}, cathepsin E or cathepsin E siRNA (Fig. 16). After stimulation with antibody 557 for 20 hours, migration was impaired in L1-transfected cells upon cathepsin E depletion, in cells carrying mutant L1, and in cells co-transfected with cathepsin E and mutant L1 when compared to non-stimulated HEK cells carrying L1 (control cells). Migration was not altered in cells transfected with cathepsin E or cathepsin E siRNA alone in comparison to non-transfected HEK cells (data not shown). In contrast, stimulated HEK cells carrying L1 only as well as cells co-transfected with L1 and cathepsin E showed significantly higher migration rates than control cells, indicating that cathepsin E activity on L1 at E_{1167} leads to an enhanced cell migration (Fig. 16).

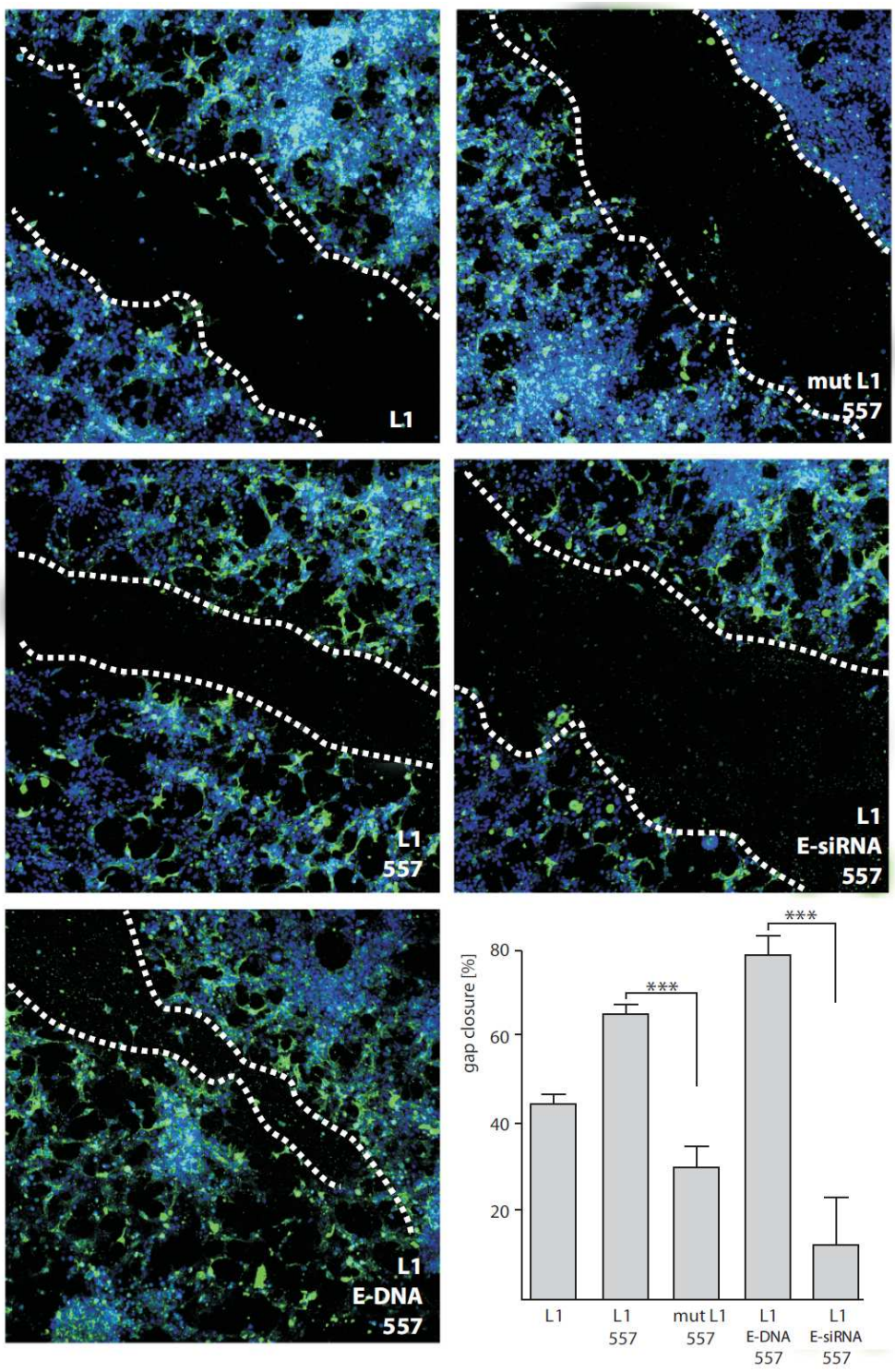

FIGURE 16. **Cathepsin E cleavage of L1 at E_{1167} modulates migration of HEK cells after scratch injury.** Overlays of representative fluorescent confocal images showing gap closure after 20 h stimulation with antibody 557; cells were stained with DAPI (blue) and L1 antibody (green), gap borders are emphasized with dashed lines. HEK cells were transfected with L1, L1 mutated at E_{1167} (mut L1), L1 and cathepsin E (E-DNA), L1 and cathepsin E siRNA (E-siRNA) and stimulated with antibody 557 for 20 h, *Graph:* Evaluation of gap

closure after stimulation with antibody 557 for 20 hours: Migration was impaired in L1-transfected cells upon cathepsin E depletion, in cells carrying mutant L1 when compared to non-stimulated HEK cells transfected with L1. In contrast, stimulated HEK cells transfected with L1 only as well as cells co-transfected with L1 and cathepsin E showed significantly higher migration rates than non-stimulated cells expressing L1 alone. Six coverslips from two independent experiments were analyzed per condition. Two images per coverslip were taken and 10 distances between the gap borders from each image were measured (*** $p<0.005$, one-way-ANOVA with Tukey's Multiple Comparison Test).

The combined results from the experiments with cerebellar explants and transfected HEK cells demonstrate clearly that generation of the 30 kDa L1 fragment upon cathepsin E activity at E_{1167} is important for neuronal cell migration.

Discussion

This work emphasizes one essential role of L1: modulation of neural morphogenic functions not as a static adhesion molecule, but rather as a signalling facilitator/transducer acting in the form of proteolytic fragments which undergo remarkable intracellular dynamics during their trafficking. The main focus of this work was to study the intracellular fate of L1 proteolytic fragments which might trigger signalling pathways to modulate fundamental neuronal cell responses, such as neuritogenesis and cell migration.

Recognition between L1 and its homophilic and heterophilic partners, which occurs at the cell surface (Werz and Schachner, 1988; Appel et al., 1993), is the first step in a complex series of highly dynamic intracellular events, such as changes in the steady-state levels of intracellular messengers and in the activities of protein kinases (Schuch et al., 1989; Atashi et al., 1992; Von Bohlen Und Halbach et al., 1992; Appel et al., 1995). Changes in levels of messengers such as inositol phosphates, Ca^{2+}, pH and cyclic nucleotides as well as changes in levels of G proteins are tightly connected with L1-mediated neurite outgrowth (Williams et al., 1992). Antibodies have been developed in order to specifically stimulate L1 involved in such events and, thus, to establish the linkage of cell surface recognition during neuritogenesis to signal transduction (Appel et al., 1995; Chen et al., 1999).

L1-specific Stimulation with Antibody 557

The antibody 557 has been found to recognize a sequence (amino acids 818-832) at the beginning of the 3rd FNIII repeat of L1 and to promote neurite outgrowth. Other developed antibodies (555, 560.5, A4.226 etc.) are not able to trigger neuritogenesis (Appel et al., 1995).

In addition to its specifity for L1, the advantage of using antibody 557 for treatment of cells might be facilitated by its ability to mimic homophilic L1 interactions and, thereby, to promote neural responses such as neuritogenesis, glial process formation, migration and neuronal survival. In contrast to antibody 557, the application of a fusion protein containing the extracellular domain of L1 and human Fc (L1-Fc) would mimic both homophilic and heterophilic interactions of L1 with other adhesion molecules. Choosing therefore antibody 557 for application might allow L1-mediated events to occur without stimulation of further cell recognition molecules. The effects that emerge upon stimulation with antibody 557 can be then directly referred to L1.

Stimulation of cultured cerebellar neurons by function triggering antibody 557 leads to generation and nuclear transport of an unknown L1 fragment with an apparent molecular weight of 70 kDa. The 70 kDa fragment is not only recognized by antibody 557 but also by antibody 172-R directed against an intracellular moiety of L1, indicating that the fragment contains the intracellular and transmembrane domains as well as a part of the extracellular domain of L1. This is the first proposal for a transmembrane L1 fragment with an extracellular moiety, which is translocated from the cell surface into the nucleus upon antibody stimulation.

L1 stimulation leads not only to generation of a 70 kDa L1 fragment, but also to generation of a corresponding 135 kDa fragment, which is released into the cell culture supernatant. Since the serine protease inhibitor aprotinin inhibits generation of the 70 kDa fragment and its corresponding 135 kDa fragment, and 557 antibody recognizes only the 70 kDa, but not the 135 kDa fragment,

these fragments must differ from the previously described 80 and 140 kDa fragments (see Fig. 1; Sadoul et al., 1988; Nayeem et al. 1999; Silletti et al., 2000; Kalus et al., 2003). Despite the smaller molecular weight, the 70 kDa fragment contains a larger part of full-length L1 in comparison to the 80 kDa fragment. To explain this discrepancy, the novel results from this thesis have to be included in Fig. 1 in order to precisely render how different L1 fragments are generated (see Fig. 17). The previously described 80 kDa L1 fragment seems to be a stable dimer of two 40 kDa L1 fragments containing the intracellular domain and an extracellular stump of L1 including 5th, 4th and a part of the 3rd FNIII domain, whereas the 70 kDa fragment comprises a monosumoylated 55 kDa L1 fragment consisting of the intracellular domain and four FNIII repeats of L1 (Fig. 17B,G). Dimerization has been shown for a variety of other recognition molecules, such as cadherins, integrins and selectins to be important for signal transduction (Tamura et al., 1998; Kalus et al., 2003). Dimers are stable even under SDS denaturating conditions and it faces very harsh conditions to split them into monomers, so that dimerized fragments cannot be easily discriminated from monomers with similar apparent molecular weight. Nevertheless, L1 proteolytic fragments can be distinguished after detection with antibodies recognizing different regions of L1: antibody 172-R is directed against the intracellular domain of L1, antibody 555 can recognize an amino acid sequence (amino acids 810-824) within the 2nd FNIII domain, antibody 557 is raised against the sequence at the N-teminal part of the 3rd FNIII domain of L1, and the polyclonal L1 antibody is raised against the entire soluble extracellular domain of L1.

Full-length L1 (L1-200, Fig. 17A) can be recognized by four different antibodies: 172-R, 555, 557 and polyclonal L1 antibody. Dimerized full-length L1 is cleaved by PC5A generating a membrane-bound 80 kDa fragment

(dimer of two 40 kDa fragments) and two 140 kDa monomers that are shed into the extracelluar space (Fig. 17B,C). Since PC5A preferencially cleaves a sequence within the 3rd FNIII repeat (amino acids 840-845), which is membrane proximally to the recognition sites for antibodies 555 and 557, the 80 kDa fragment can be detected only with antibody 172-R and the polyclonal L1 antibody. After cleavage within this PC5A sequence, the length of the remnant corresponds to a molecular weight of approximately 40 kDa. Presumably, the 80 kDa fragment appears to be a dimer of two 40 kDa remnants. One L1-200 can be processed by PC5A to a soluble 140 kDa fragment (monomer) which is recognized by the antibodies 555, 557 and polyclonal L1 (Fig. 17C) and released in the extracellular space. The 80 kDa fragment can be further processed by ADAM10 to a 32 kDa fragment which similarly to L1-80 represents a stable dimer of two 16 kDa fragments (Fig. 17D) recognized only by antibody 172-R. If PC5A and ADAM10 cleave the 80 kDa fragment, the appearing 50 kDa fragment will be recognized only by the polyclonal L1 antibody, for it is lacking the recognition sequences for antibodies 172-R, 555 and 557 (Fig. 17E). The 50 kDa fragment represents a dimer of two 25 kDa L1 fragments. Subsequent cleavage of the 32 kDa fragment leads to generation of a 28 kDa fragment (dimer) which by implication must comprise two intracellular domains of L1 (Fig. 17F). This fragment is recognized only by antibody 172-R.

The proteases trypsin, plasmin, and PC5A, which cleave L1 distally to the binding site of the antibody 557 at the N terminus of the third FNIII domain, cannot be involved in generation of the 70 and 135 kDa fragments seen in cerebellar neurons and their supernatants after L1 stimulation. These fragments must originate from the activity of a further, yet unknown, serine protease (Fig. 17G,H) which is sensitive to aprotinin. The 135 kDa fragment is recognized

only by the polyclonal L1 antibody, since cleavage of L1 by a serine protease generates a fragment lacking the recognition sites for antibodies 555 and 557. The 70 kDa fragment is detectable with all four antibodies: 172-R, 555, 557 and polyclonal L1 antibody indicating that the fragment contains the 2nd, 3rd, 4th and 5th FNIII repeats. Therefore, the 70 kDa fragment comprises a longer sequence of L1 than a 40 kDa monomer of the 80 kDa L1 fragment. In addition, the 70 kDa fragment is sumoylated. Regarding the apparent molecular weight of sumo, the 70 kDa fragment might be a combination of a 55 kDa L1 fragment and a 15 kDa sumo-protein.

Sumoylation is Required for Proteolytic Cleavage of L1 at the Plasma Membrane

L1 contains two putative monopartite nuclear localization signals (K_{1147}RSK and K_{1235}KEK) and two potential sites for attachment of small ubiquitin-like modifier proteins (GK_{1235}KE and MK_{1172}DE). Site-directed mutagenesis of the sumoylation site at K_{1172} abolishes L1-stimulated generation of the 70 kDa fragment. Mutagenesis of the nuclear localization signal at K_{1147} inhibits nuclear import of this fragment, whereas alteration of the K_{1235}KEK motif affects neither generation nor nuclear import of this fragment or of the 70 kDa fragment. Furthermore, Western blot analysis of HEK293 cells co-transfected with L1 and GFP-tagged sumo-1, sumo-2 or sumo-3 reveals GFP-positive L1 bands at 100 kDa after stimulation with antibody 557, indicating that attachment of one GFP-tagged sumo (45 kDa) to a 55 kDa L1 fragment shifts its molecular weight to 100 kDa – monosumoylated 55 kDa L1 fragment represents the novel 70 kDa L1 fragment described in this thesis. Taken together, these findings suggest that monosumoylation of K_{1172} is required for L1 proteolytic processing at the plasma membrane which leads to generation of the 70 kDa fragment (Fig. 18, step 1). Since the apparent molecular weight of

one sumo has been reported to be ~15 kDa (Shiio and Eisenman, 2003), it was concludable that the 70 kDa fragment represents a monosumoylated 55 kDa L1 fragment indeed.

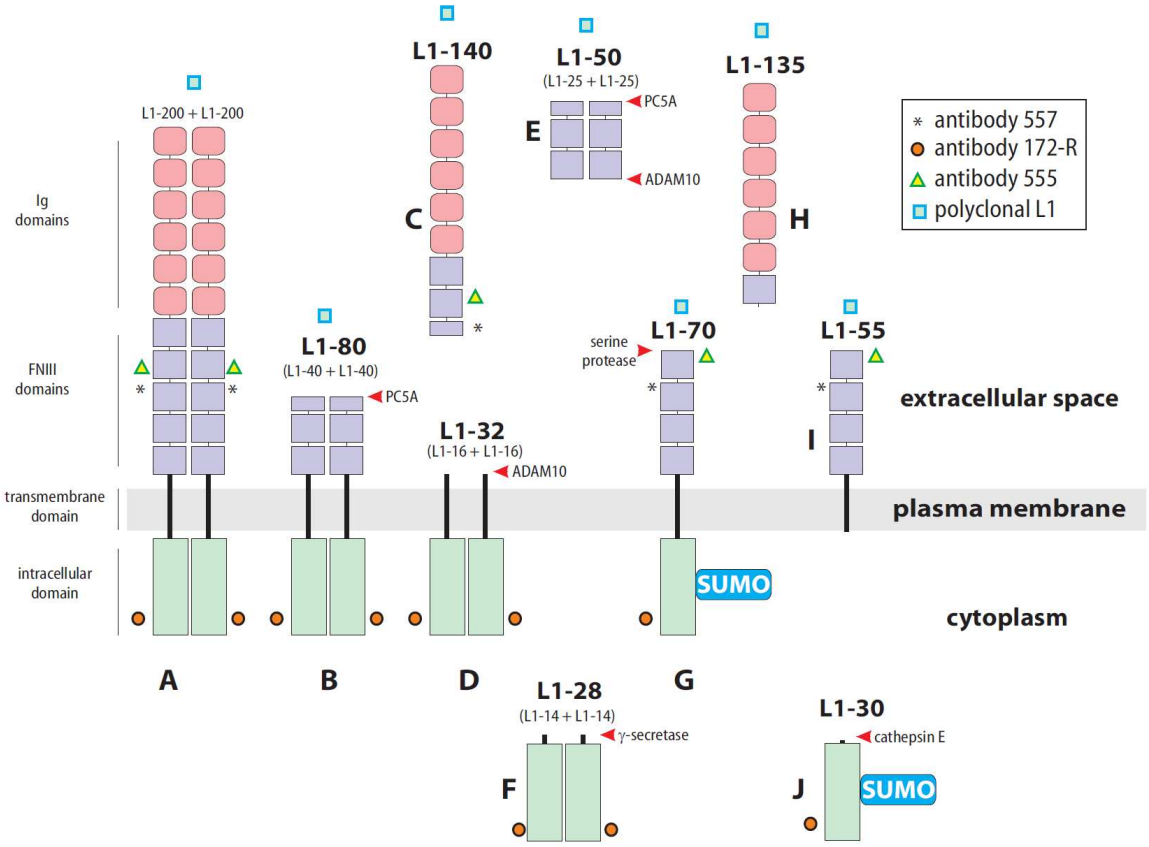

FIGURE 17. **Schematic representation of different proteolytic L1 fragments based on their structure and recognizing antibodies.** *A*, full-length L1 (L1-200) is recognized by four different antibodies: 172-R, 555, 557 and polyclonal L1. *B*, in dimerized form, L1-200 is processed by PC5A generating a membrane-bound 80 kDa fragment (L1-80) which is recognized only by antibody 172-R and polyclonal L1 antibody and a shedded soluble 140 kDa fragment (*C*). Since PC5A cleaves L1-200 in its 3rd FNIII repeat and the size of the resulting L1 fragment is approximately 40 kDa, L1-80 comprises two L1-40 fragments. *C*, one L1-200 can be processed to a soluble 140 kDa fragment which is recognized by the antibodies 555, 557 and polyclonal L1. *D*, L1-80 can be further cleaved by ADAM10 to a 32 kDa fragment which similarly to L1-80 represents a stable dimer consisting of two 16 kDa fragments (L1-16) recognized only by antibody 172-R. *E*, if PC5A and ADAM10 consecutively cleave L1-80, the appearing 50 kDa fragment (L1-50) would be recognized only by the polyclonal L1 antibody. L1-50 represents a dimer of an L1-25 form which is lacking the recognition sites for the 555 and 557 antibodies. *F*, subsequent cleavage of L1-32 leads to generation of a 28 kDa fragment (L1-28, *F*) which comprises two intracellular domains of L1 (L1-14). *F*, L1-28 is recognized only by antibody 172-R. *G*, a serine protease generates the novel 70 kDa fragment (L1-70) and the 135 kDa (L1-135, *H*) fragment. L1-70 is sumoylated and detectable with all four antibodies: 172-R, 555, 557 and polyclonal L1. *H*, L1-135 is recognized only by the polyclonal L1 antibody, as the serine

protease removes the recognition sites for the antibodies 555 and 557. *I,J*, cathepsin E cleaves sumoylated L1-70 to generate an intracellular 30 kDa (L1-30, *J*) and a membrane-bound 55 kDa (L1-55, *I*) fragment. *I*, in contrast to the soluble L1-50 (*E*), the membrane-bound L1-55 (*I*) can be recognized by three antibodies: 555, 557 and polyclonal L1. *J*, in contrast to the intracellular L1-28 (*F*), the intracellular L1-30 (*J*) consists of only one intracellular domain of L1 which is sumoylated. Similarly to L1-32 (*D*) and L1-28 (*F*), antibody 172-R recognizes L1-30 (*J*) as well.

In summary, L1 can exist in diverse proteolytic forms, which are derived from a controlled cell surface phenomenon termed ectodomain shedding. Ectodomain shedding and receptor endocytosis are emerging mechanisms in cell motility. Ectodomain shedding is a proteolytic mechanism by which transmembrane molecules are converted into a soluble form and which has been shown for a variety of molecules, such as growth factors, growth factor receptors, cadherins, selectins and other Ig-CAMs (Reiss et al., 2006). The phenomenon of ectodomain shedding is an important tool for reassembling the cell surface composition and architecture. An increasing body of evidence shows that not only fragments, but also full-length single spanning transmembrane cell surface receptors are translocated to the nucleus (Planque, 2006). For instance, the entire EGF receptor appears to be relocated from the plasma membrane to the ER, and is released from there into the cytoplasm to be further transported into the nucleus in an importin- and hsp70-dependent manner (Liao and Carpenter, 2007).

Due to the presence of different parts of full-length L1 in the different proteolytic fragments, these forms are expected to vary tremendeously in their functions: different epitopes facilitate distinct interactions with other proteins; variations of fragment size modulates distinct intracellular diffusivity of the fragments; sumoylated fragments might be tailored with a wider interaction repertoire than non-sumoylated fragments, and the potential interaction surface of dimers is obviously larger than that of monomers. Moreover, the

intracellular fate of the fragments might be shaped by the vast scope of possibilities for interaction with different binding partners involved in numerous signalling cascades.

Intracellular Trafficking of the 70 kDa L1 Fragment

After generation at the plasma membrane (Fig. 18, step 1), the sumoylated 70 kDa L1 fragment is internalized by endocytosis (Fig. 18, step 2) to be distributed via vesicles to the late endosomal or MVB compartment and to the ER (Fig. 18, steps 3,3a,3b). Western blot analysis of fractions isolated from plasma membrane, cytoplasm, endosomes and nucleus containing the proteolytically generated, sumoylated and biotinylated 70 kDa L1 fragment confirms that this fragment is trafficked from the plasma membrane to the nucleus via endosomes and the cytoplasm. The endosomal-cytosolic path of the 70 kDa L1 fragment seems to depend on ESCRT-III-associated proteins (CHMP1, Alix and Vps4), as antibodies against these escort proteins, but not against the ESCRT-I subunit Tsg101, abolish release of the fragment from endosomal membranes in the retranslocation assay. Furthermore, nuclear import of the 70 kDa L1 fragment is blocked in the presence of antibodies against importin and CHPM1, whereas antibodies against Vps4 and Alix or control antibodies do not impair the import of the fragment. Interestingly, upon L1 stimulation of cerebellar neurons the level of DNA-associated CHMP1 increases as the level of membrane-bound CHMP1 decreases and the level of cytoplasmic CHMP1 remains constant. The combined results provide evidence that redistribution of the 70 kDa L1 fragment from the cytoplasm to the nucleus is accompanied by altered subcellular localization and nuclear import of CHMP1. CHMP1 has been shown not only to be implicated in MVB formation (Howard et al., 2001), but also to be present in interphase nuclear matrix and mitotic chromosome scaffold. In addition, overexpression

of CHMP1 strongly affects nuclear structure and DNA replication (Stauffer et al., 2001). The association of the L1 fragment with CHMP1 for nuclear import and its presence in chromatin-bound fractions suggest possible functional roles of the 70 kDa fragment in modulating gene expression via direct or indirect interaction with DNA and/or transcription factors, nuclear signalling, cellular and subcellular reorganization, and plasticity.

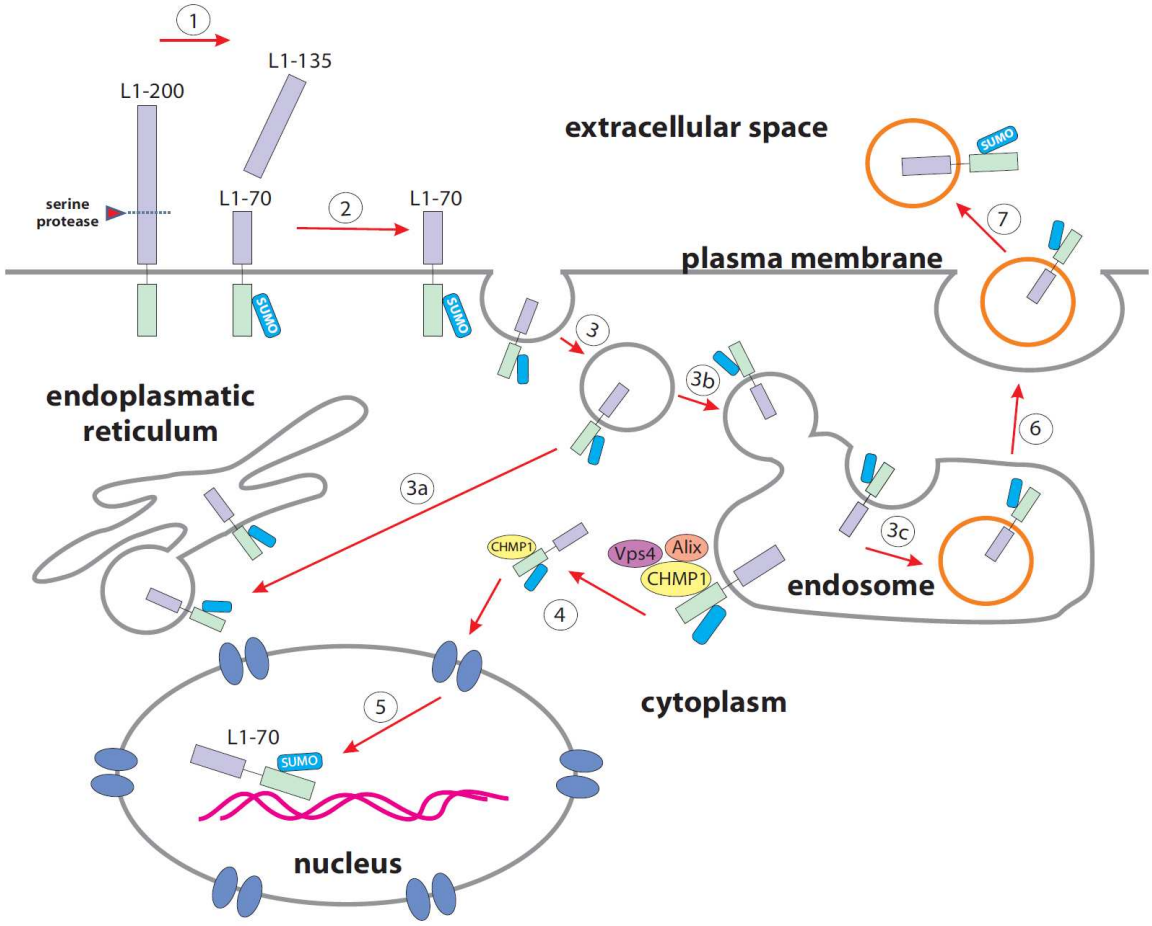

FIGURE 18. **Working model for generation and intracellular trafficking of the transmembrane 70 kDa L1 fragment (L1-70).** *1*, ectodomain shedding of full length L1 (L1-200) by an unknown serine protease results in generation of the membrane-bound 70 kDa L1 fragment (L1-70) and its corresponding soluble 135 kDa fragment (L1-135); *2,3*, L1-70 is internalized in neurons via endocytosis; *3a,3b*, endosomes distribute L1-70 to the endoplasmatic reticulum, the sorting endosomes and late endosomes; *3c*, formation of MVBs carrying L1-70; *4*, after release from the endosomes which depends on the ESCRT-III-proteins Alix, Vps4 and CHMP1, L1-70 is imported together with CHMP1 into the nucleus; *5*, chromatin bound fractions are enriched in L1-70 which may interact with DNA

to regulate gene expression; *6*, – L1-70 can also be exocytosed for release into the extracellular space via exosomes (*7*).

Functional Role of the 70 kDa L1 Fragment

Because L1-induced neurite outgrowth is inhibited by aprotinin, which also prevents generation of the 70 kDa L1 fragment and its nuclear import, this fragment could play an important role in neuritogenesis and, thus, in development of the nervous system. In fact, expression of the 70 kDa L1 fragment in mouse brains is enhanced between postnatal day 0 and day 5 (Fig. 9), suggesting a potential role of the fragment in developmental events taking place at this time, such as axonal pathfinding and early synaptogenesis. Increased levels of the L1 fragment in segments of the thoracic spinal cord rostral from the lesion site after spinal cord lesion and in nerve segments after femoral nerve injury (Fig. 10A) suggest a possible role of the fragment in regeneration of the injured nervous system.

Given that increased amounts of soluble L1 fragments in cerebrospinal fluid are associated with Alzheimer's disease (Strekalova et al., 2006), APPPS1-21 mice appear to be an interesting model for determination of the expression levels of the 70 kDa L1 fragment. APPPS1-21 mice which develop amyloid plaques due to overexpression of the mutated form of amyloid precursor protein and increased γ-secretase activity (Radde et al., 2006) show a significant decrease in levels of the 70 kDa fragment (Fig. 10B). Since increased amounts of soluble L1 fragments in cerebrospinal fluid are associated with Alzheimer's disease and dementia syndromes in patients (Poltorak et al., 1995; Strekalova et al., 2006) and considering the altered levels of the 70 kDa L1 fragment in APPPS1-21 mouse brain homogenates, it is likely that proteolytic processing of L1 is

involved in L1-dependent cellular responses to acute and chronic damage of the nervous system.

Generation of the 30 kDa L1 Fragment from the 70 kDa Fragment

The 70 kDa L1 fragment seems to be a substrate of a pepstatin sensitive protease which generates a 30 kDa L1 fragment. Neither presenilin/γ-secretase nor metalloproteases are involved in the generation of this fragment, since inhibition of these proteases does not abolish its generation. The smaller fragment contains only the intracellular domain of L1 (Fig. 17J) and differs from the previously described intracellular fragment (Fig. 17F) originating upon metalloprotease- and presenilin/γ-secretase-mediated cleavage (Gutwein et al., 2003; Maretzky et al., 2005; Riedle et al., 2009), as the aspartyl protease inhibitor pepstatin abolishes its genesis and the generation of its corresponding 55 kDa fragment (Fig. 17I) while the 70 kDa (Fig. 17G) and its corresponding 135 kDa (Fig. 17H) fragments accumulate. Regarding this and since L1 acitvates cathepsin expression via Erk (Silletti et al., 2004), cathepsin E and D as apartyl proteases appear to be prominent candidates for generation of the 30 kDa L1 fragment upon L1 specific stimulation with antibody 557. Although highly homologous, cathepsin E and D differ in their subcellular localization. Cathepsin D is predominantly found in lysosomes, whereas cathepsin E has been found in endosomes, endoplasmic reticulum, Golgi apparatus and at the plasma membrane (Zaidi et al., 2008). Moreover, cathepsin E has been detected at the cytoplasmic surface of the plasma membrane (Ueno et al., 1989) and is evenly distributed within the cytoplasm (Saku et al., 1990; Hara et al., 1993).

Cathepsin E Cleaves the 70 kDa Fragment to Generate the 30 kDa Fragment

Cathepsin E preferentially cleaves Glu-Ala (EA) bonds at physiological pH (Athauda et al., 1991). Due to the fact that all known mammalian L1 family molecules and homologs contain an EA sequence adjacent to the sumoylation site MKDE within the intracellular domain, cathepsin E can be speculated to cleave the sumoylated 70 kDa fragment at this site generating the 30 kDa fragment. Gene silencing of cathepsin E which prevents appearance of the 30 kDa L1 fragment without changing the levels of the 70 kDa L1 fragment in lysates from cerebellar neurons strongly supports this view. In addition, site-directed mutagenesis of E_{1167} to Q_{1167} impairs the generation of the 30 kDa fragment revealing that this fragment and its corresponding 55 kDa fragment originate from the 70 kDa fragment upon cathepsin E activity (Fig. 19).

Western blot analysis of different subcellular fractions indicates that the membrane-associated 55 kDa fragment is internalized by endocytosis after its generation at the plasma membrane, transported to the late endosomes/MVBs or lysosomes, transferred to exosomes and released into the extracellular space in association with exosomes (Fig. 19, steps 2,5,6,7,8,9). Exosomes are membrane vesicles released from a score of different cell types including tumor cells, red blood cells, platelets, lymphocytes and dendritic cells (Andre et al., 2002; Thery et al., 2002). Exosomes are formed by invagination and budding from the limiting membrane from late endosomes (Raiborg et al., 2003). They accumulate in cytosolic MVBs from where they are released by fusion with the plasma membrane (Raiborg et al., 2003). Exosomes can recruit various cytosolic and plasma membrane proteins: MHC molecules, tetraspanins, ashesion molecules and metalloproteases (Andre et al., 2002; Thery et al., 2002). Although believed to be a cell-surface event, ectodomain shedding can

occur also intracellularly, where exosomes serve as its platform and as a vehicle for cellular export of soluble molecules (Stoeck et al., 2006). Due to their enrichment in proteolytic and adhesive activity, exosomes released from tumor cells are speculated to promote cellular invasion and migration during metastasis (Dolo et al., 1998; Ginestra et al., 1998).

It can be further speculated, that exosomes carrying L1 fragments might contribute to a long-distance crosstalk for activating of complex signalling in and between tissues. However, it remains unknown whether the exosome-bound 55 kDa L1 fragment which is released into the cell culture supernatant upon L1 stimulation with antibody 557 is of functional relevance in these processes.

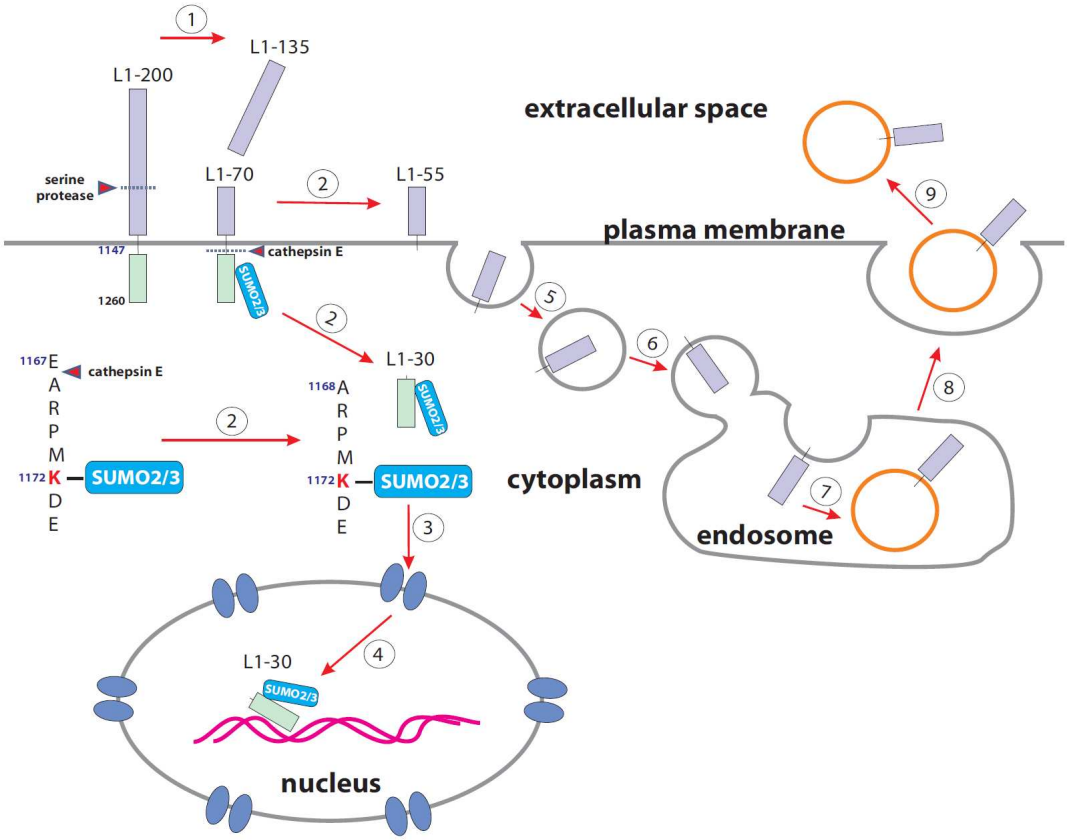

FIGURE 19. **Working model for generation and intracellular trafficking of the 30 kDa L1 fragment (L1-30).** *1*, after generation from full length L1, L1-70 sumoylated with sumo-2

and/or sumo-3 is a substrate for cathepsin E. *2*, cathepsin E cleaves L1-70 at E_{1167} to generate L1-30 and its corresponding fragment L1-55. The latter remains bound to the membrane, while L1-30 is released into the cytoplasm. *3,4*, after release into the cytoplasm, L1-30 is relocated into the nucleus where it may interact with DNA similarly to L1-70. *5*, L1-55 is distributed to the late endosomes. *6*, after formation of MVBs, L1-55 is released into the extracellular space by exocytosis (*7*) and via exosomes (*8,9*).

Sumoylation and Intracellular Distribution of the 30 kDa L1 Fragment

The abundance of the 30 kDa L1 fragment in cytoplasmic and nuclear fractions, but not in association with membranes (unpublished observations), indicates that this fragment is rapidly trasported towards the nucleus after its release from the plasma membrane into the cytoplasm (Fig. 19, step 2,3,4). In addition, generation and nuclear import of the 30 kDa L1 fragment depend on sumoylation of L1 at K_{1172} and on the nuclear localization signal K_{1147}RSK, as described for the 70 kDa L1 fragment. Mutation of these motifs leads to an impaired generation and nuclear import of both fragments.

The nuclear translocation of the 30 kDa fragment may be an indication that this fragment is involved in L1-dependent nuclear signalling. Since the 30 kDa fragment is detectable in nuclear fractions containing chromatin-associated proteins, it is possible that this fragment might interact with transcription factors and/or DNA and might be involved in modulating gene expression. Here, these findings provide evidence that the 30 kDa fragment is not involved in neurite outgrowth, but that it is involved in promotion of L1-triggered neuronal migration: L1-induced generation of the 30 kDa fragment and neuronal migration are inhibited by pepstatin and, moreover, they are facilitated by the cathepsin E activity on L1 at E_{1167}.

Interestingly, cathepsin E appears to cleave the 70 kDa fragments modified with sumo-2 or -3, while it does not cleave 70 kDa fragments carrying sumo-1. Sumoylation is a post-translational modification which requires formation of an isopeptide bond between the C-terminal glycine residue of sumo and the ε-amino group of a lysine acceptor in the target protein (Mahajan et al., 1997). Although it resembles biochemically ubiquitination, both machineries do not overlap (for review, see Geiss-Friedlander and Melchior, 2007). Sumo-1 and sumo-2/3 use the same enzymatic conjugation pathway (Tatham et al., 2001), but they most probably serve different functions as the different isoforms can be conjugated to different target proteins (Saitoh and Hinchey, 2000; Vertegaal et al., 2006). Sumoylation has been shown to modify the activity, degradation, inter- or intramolecular interactions and localization of target proteins, but also to influence nucleo-cytoplasmic transport, transcription and DNA repair (Geiss-Friedlander and Melchior, 2007; Scheschonka et al., 2007). Furthermore, sumoylation plays important roles in modulating synaptic transmission and plasticity (Martin et al., 2007), neuronal cell motility and axonal guidance (Kadaré et al., 2003), and, thus, in development and function of the adult central nervous system (Martin et al., 2007; Loriol et al., 2012). Interestingly, complete abrogation of sumo conjugation leads to embryonic lethality in mice (Nacerddine et al., 2005; Martin et al., 2007). Moreover, alterations in sumoylation are linked to diseases, such as neurodegenerative disorders and cancer (Geiss-Friedlander and Melchior, 2007; Scheschonka et al., 2007). Increasing numbers of reports reveal the involvement of sumoylation in synucleinopathies, including Alzeimer's and Parkinson's diseases, as well as in polyglutamine disorders, such as Huntington's disease and spinocerebellar ataxias (Krumova and Weishaupt, 2012).

In addition, sumo-2/3 overexpression has been tightly connected with amyloid precursor protein processing (Li et al., 2003; Dorval et al., 2007) and sumoylation of amyloid precursor protein reduces amyloid-β production (Zhang and Sarge, 2008). Since the sumoylated 30 kDa L1 fragment appears to play a role in neuronal migration and since mutations in L1 and loss of function of L1 are associated with several mental and cognitive abnormalities, such as L1 syndrome, fetal alcohol syndrome, schizophrenia (Katidou et al., 2008) and autism (Melitta Schachner, personal communication) with a common denominator of impaired cell migration, the sumoylated 30 kDa L1 fragment can be proposed to be the pivotal link between these L1-linked abnormalities. Furthermore, enhanced expression of L1 correlates with enhanced migration of almost all types of tumor entities so far investigated (Kaifi et al., 2007; Schäfer and Altevogt, 2010) resulting in increased metastatic activities that correlate with intensity of metastatic spread and decreased survival rates of affected patients (Kiefel et al., 2012).

Diversity through Fragments

The present work demonstrates how L1 can gain functional diversity through its persistence in the form of proteolytic fragments and post-translational modification of these fragments. In a controlled fashion, homophilic interaction allows a transmembrane 70 kDa L1 fragment and an intracellular 30 kDa concomitant to occur. In a unique mode, these fragments are tailored to exhibit different functions. Moreover, sumoylation seems to orchestrate this mode of proteolytic processing of L1: only the 70 kDa fragment modified with sumo-2/3 is preferentially cleaved by cathepsin E to generate the 30 kDa fragment. Both fragments undergo different intracellular paths of trafficking, suggesting that sumoylation can shape the intracellular fate of proteolytic

fragments: sumoylated L1 fragments can enter the nucleus - if their sumoylation is abolished, so is nuclear import impaired as well.

In fact, the intracellular presence of proteolytic fragments can be correlated with specific functional properties. For instance, cytosolic and nuclear localization has also been observed for CD146 (also known as cell surface glycoprotein MUC18 and melanoma associated cell adhesion molecule) in endothelial progenitor cells. In particular, nuclear translocation appears to be a specific property of the short CD146 isoform. In contrast to the short isoform, the long CD146 isoform has a diffuse cytosolic pattern (Kebir et al., 2010). The different localization of the two isoforms underlies different functions, the short CD146 fragment promotes migration and proliferation of epithelial progenitor cells, and the long CD146 fragment is involved in the subsequent stabilization of capillary structures (Kebir et al., 2010).

The 30 kDa L1 fragment is involved in neuronal migration, whereas the 70 kDa fragment plays a role in neuritogenesis and is functionally implicated in development, regeneration, tumorigenesis, and possibly synaptic plasticity in the nervous system. Futhermore, the presence of both fragments in the nucleus suggests interaction with DNA and transcription factors, which might define the fragments as affectors of nuclear events, such as gene expression.

The ability of cell adhesion molecules to modulate cellular responses has been traditionally viewed as a consequence of their adhesive functions. However, upon stimulation with antibody 557, the cell adhesion molecule L1 is proteolitically cleaved and shed from the membrane. Thus, the downstream effects of stimulation appear to be a result from the emerging soluble

proteolytic fragments, which can act as triggers of signalling cascades and modulators of gene expression.

Abbreviations

ADAM	a disintegrin and metalloproteinase
Ala	alanine
Alix	apoptosis-linked gene-2-interacting protein X
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
CA3	region in the hippocampus, CA (Cornu ammonis)
CAMs	cell adhesion molecules
CD24	cluster of differentiation 24
CD146	cluster of differnetiation 146
CHMP1	chromatin-modifying protein 1
Cy	cytochrome
DAPI	4',6-diamidino-2-phenylindole
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DMEM	Dulbecco's modified Eagle's medium
DNA	desoxyribonuclein acid
DTT	dithiothreitol
ECD	extracellular domain
EGF	epidermal growth factor
ER	endoplasmic reticulum
Erk	extracellular-signal-regulated kinase
ERM	ezrin-radixin-moesin
ESCRT	endosomal sorting complex required for transport
FNIII	fibronectin type III domain
GFP	green fluorescent protein
Glu	glutamic acid
GM6001	(2R)-N'-hydroxy-N-[(2S)-3-(5H-indol-3-yl)-1-methylamino-1-oxopropan-2-yl]-2-(2-methylpropyl)butanediamide
HEK	human embryonic kidney

HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HP1γ	heterochromatin-associated protein 1-γ
ICD	intracellular domain
Ig	immunoglobulin
IgG	immunoglobulin G
kDa	kilodalton
L1	cell adhesion molecule L1
L1-Fc	hybrids of the extracellular domain of the cell adhesion molecule L1 and human Fc
L1-KO	knockout of the L1 gene
Lys	lysine
MASA	mental retardation, aphasia, shuffling gait, adducted thumbs
MVB	multivesicular body
NCAM	neuronal cell adhesion molecule
NLS	nuclear localization signal
PBS	phosphate buffered saline
PC5A	pro-protein convertase
PLL	poly-L-lysine
PMSF	phenylmethylsulfonyl fluoride
RIP	regulated intramembrane proteolysis
SDS	sodium dodecyl sulfate
siRNA	small interfering ribonuclein acid
SUMO	small ubiquitin-like modifier
Tsg101	tumor susceptibility gene 101
Vps4	vacuolar protein sorting-associated protein 4
WB	Western blot

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