Distinct Intracellular Domain Substrate Modifications Selectively Regulate Ectodomain Cleavage of NRG1 or CD44

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Ectodomain cleavage by A-disintegrin and -metalloproteases (ADAMs) releases many important biologically active substrates and is therefore tightly controlled. Part of the regulation occurs on the level of the enzymes and affects their cell surface abundance and catalytic activity. ADAM-dependent proteolysis occurs outside the plasma membrane but is mostly controlled by intracellular signals. However, the intracellular domains (ICDs) of ADAM10 and -17 can be removed without consequences for induced cleavage, and so far it is unclear how intracellular signals address cleavage. We therefore explored whether substrates themselves could be chosen for proteolysis via ICD modification. We report here that CD44 (ADAM10 substrate), a receptor tyrosine kinase (RTK) coreceptor required for cellular migration, and pro-NRG1 (ADAM17 substrate), which releases the epidermal growth factor (EGF) ligand neuregulin required for axonal outgrowth and myelination, are indeed posttranslationally modified at their ICDs. Tetradecanoyl phorbol acetate (TPA)-induced CD44 cleavage requires dephosphorylation of ICD serine 291, while induced neuregulin release depends on the phosphorylation of several NRG1-ICD serines, in part mediated by protein kinase C δ (PKC δ). Downregulation of PKC δ inhibits neuregulin release and reduces *ex vivo* neurite outgrowth and myelination of trigeminal ganglion explants. Our results suggest that specific selection among numerous substrates of a given ADAM is determined by ICD modification of the substrate.

Many transmembrane proteins on the cell surface are subject to proteolytic cleavage of their ectodomains, predominantly by metalloproteases (ectodomain shedding) (1–3). Ectodomain shedding regulates numerous important molecules involved in signal transfer between the extracellular space and the cell's interior and thus influences many cellular functions (1, 3). This includes, for example, the biological availability of epidermal growth factor (EGF) receptor ligands such as neuregulin (NRG1) (4, 5) and the modulation of complex cellular phenotypes required for contact inhibition of cells involving the hyaluronic acid receptor CD44 (4). NRG1 regulates neurite outgrowth and myelination but also has important functions in the development of other organs, for instance, the heart (6–9). When bound to hyaluronan, CD44 triggers a proliferation-inhibitory pathway (10–12). On the other hand, cancer stem cells carry CD44 (13–15), and, in this context, CD44 promotes tumor growth and metastasis (16–21), likely via alternative splice forms of CD44 that act as growth factor-enriching coreceptors for receptor tyrosine kinases (RTKs) (22, 23).

Inappropriate proteolysis of a number of shed substrates is associated with diseases when cleavage is either upregulated or reduced (24, 25). Equally, total knockout of substrates leads to significant phenotypes (26, 27). This indicates that ectodomain cleavage requires tight regulation. How ectodomain cleavage is regulated and made substrate specific is largely unknown to date.

The metalloproteases ADAM10 and ADAM17 are involved in the cleavage of most substrates that undergo regulated cleavage induced by intracellular signaling pathways, which are, in turn, activated by G protein-coupled receptors (GPCRs) or RTKs (2) involving the activation of protein kinase C (PKC) isoforms (5, 28, 29). An obvious way to regulate cleavage is modulation of the availability and activity of the enzymes. Indeed, ADAMs (A-disintegrin and -metalloproteases), in particular ADAM17, are regulated by several mechanisms that affect their activity, including

the level of their expression, trafficking from intracellular compartments to the cell surface (their site of action), removal of the inhibitory prodomain (reviewed in reference 2), and modulation of their catalytic ectodomain structure (30). The last can involve redox regulation targeted to the outside of the cell that induces irreversible changes in the ADAM17 membrane-proximal CANDIS domain relevant for interaction with some select ADAM17 substrates (31–33). C-terminal phosphorylation of ADAM17 has been reported to increase its surface levels and releases ADAM17 dimers from their inhibitory interaction with the extracellular inhibitor TIMP3 to form presumably active monomers (34, 35).

We along with others have provided evidence that ectodomain cleavage is also regulated on the substrate level by C-terminal modification of the substrate (5). Release of neuregulin from its precursor NRG1 requires phosphorylation at serine 286 by PKC δ

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(5). CD44 cleavage is specifically regulated by the tumor suppressor merlin (4).

Here, we provide extended and detailed evidence for specific regulation of the cleavage of NRG1 (ADAM17 substrate) and CD44 (ADAM10 substrate) by C-terminal modification involving different PKC isoforms and the *in vivo* relevance of these ICD modifications. Using chimeric proteins, we show that it is the substrate's C terminus that determines cleavage, independent of the ectodomain involved. In trigeminal ganglion explants, we show that C-terminal cleavage regulation of NRG1 is important for neuronal outgrowth and myelination *in vivo*. C-terminal inhibition of CD44 cleavage by merlin, in turn, blocks migration of cancer cells, linking CD44 cleavage regulation by merlin to a cancer-relevant phenotype (4).

MATERIALS AND METHODS

Reagents. The following were used: DNA oligonucleotides (Metabion), tetradecanoyl phorbol acetate (TPA), DAPT, angiotensin II (AngII), trypsin, chymotrypsin, and trichloroacetic acid-deoxycholate (TCA-DOC) (Sigma); beta-secretase (BACE) inhibitor IV, okadaic acid, Gö6976, bis-indolylmaleimide I (BIM1) and batimastat (BB94) (Calbiochem); GM-6001 and compound E (Enzo); soluble ADAM10 catalytic domain and soluble NRG1 (R&D Systems); 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories); EZ Link sulfo-NHS-LC-LC-biotin (sulfosuccinimidyl-6-[biotinamido]-6-hexanamido hexanoate; Thermo Scientific); streptavidin-Sepharose and Lipofectamine 2000 (Invitrogen); Eugene 6 and complete protease inhibitor cocktail (Roche).

Antibodies. The following antibodies were used: anti-FLAG (M2 and SIG1-25) (Sigma); ADAM10 (amino acids 735 to 749) and ADAM17 (TACE amino acids 807 to 823) (Calbiochem or R&D Systems); another ADAM17 C-terminal antibody (gift of Carl Blobel, Hospital for Special Surgery, New York, NY); moesin (Ab-1) (Thermo Fisher Scientific); c-Myc (9E10), hemagglutinin (HA; F-7), green fluorescent protein (GFP; B-2), moesin (C-15), NF2 (merlin) (C-19, C-18, and B-12), c-Met (C-12), neuregulin-1 α / β 1/2 (C20), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), S-100 β (N-15), and actin (I-19) (Santa Cruz Biotechnology); PKC δ (D10E2) and GFP (4B10 and D5.1) (Cell Signaling Technology); α -tubulin (ab4074) and Tuj-1 (β -tubulin III) (t8578) (Abcam). Secondary antibodies were the following: goat anti-rabbit IgG and anti-mouse IgG conjugated to Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647; Alexa Fluor 647-phalloidin and rhodamine-phalloidin conjugates for F-actin staining; and wheat germ agglutinin (WGA; Molecular Probes). Other secondary antibodies were from Dako (Carpinteria, CA).

Plasmids. pcDNA3.1 (Invitrogen)-based plasmids expressing cDNAs encoding human CD44, the human merlin mutants (NF2 S518A and NF2 S518D) (12), and HA-tagged bovine ADAM10 have been described previously (37). Two different ADAM10 mutants with intracellular domain (ICD) deletions were generated by site-directed mutagenesis. The sequence encoding the standard isoform of rat CD44 was subcloned into the NotI/XbaI sites of pFLAG-myc-CMV-21 (where CMV is cytomegalovirus) so that the CD44-encoding sequence was flanked by an N-terminal FLAG tag and a C-terminal myc epitope. CD44 mutants were generated by site-directed mutagenesis. Plasmids encoding mouse Flag-pro-NRG1-enhanced GFP (EGFP) and HA-transforming growth factor α (TGF- α)-EGFP have been described elsewhere (36). NRG1 ICD mutants were generated by site-directed mutagenesis; generation of the S286A mutant was described by Dang et al. (5). The sequence encoding FLAG-tagged NRG1 was also subcloned into the EcoRI and XhoI restriction sites of the pFLAG-myc-CMV-21 expression vector (Sigma). An XhoI restriction site was introduced into pFLAG-myc-CMV-21 by site-directed mutagenesis using the following primers: 5'-CTGATATCGGTACCACTCGAGTCTA GAGAACAAAACTC-3' and 5'-GAGTTTGTCTCTAGACTCGAG TGGTACCGATATCAG-3'. NRG1E/CD44(TM+ICD) (containing the ectodomain of NRG1 [NRG1E] and the transmembrane domain [TM]

and ICD of CD44) was generated by subcloning the murine NRG1 ectodomain sequence into pFLAG-myc-CMV-21 containing CD44 such that the CD44 ectodomain was replaced. Cytoplasmic mutations were introduced to NRG1E/CD44(TM+ICD) by site-directed mutagenesis as described above. CD44E/NRG1(TM+ICD) was generated in an analogous fashion. The following primer pairs (forward 5' to 3' and reverse 5' to 3') were used to generate the following constructs: ADAM10 ICD DEL1, CATGTTAATGGCTGGTTTATTTTATTTGATGTACCCG TAC (forward) and GTACGGGTACATCAAAATAAAACCAGCCATT AACATG (reverse); ADAM10 ICD DEL2, GGTTTTATTAAGATATG CAGTTAACATACTCCAAGTAG (forward) and CTACTTGGAG TATGTTAACTGCATATCTTAATAAAACC (reverse); CD44KR-MT step 1, GAAGAAGGTGTGGGCAGGCGGCAGCGCTAGTGATCAA CAG (forward) and CTGTTGATCACTAGCGCTGCCGCTGCCCA CACCTTCTTC (reverse); CD44KR-MT step 2, TTGCAGTCAACAGTC GAGCAGCGTGTGGGCAGGCGGCAG (forward) and CTCGCCCT GCCCACACGCTGCTCGACTGTTGACTGCAA (reverse); CD44 Δ ICD, GACCTCAGATTCCAGAGAACAGTAGGAGAAGGTG (forward) and CACCTTCTCCTACTGTCTCTGGAATCTGAGGTC (reverse); CD44 Δ afterKR, CAGTAGGGCAGCGTGTGGGCAGGCGGCAGCGCTGGT GATC (forward) and GATCACCAGCGCCGCCGCTGCCACAC GCTGCCCTACTG (reverse); CD44 S291A, TCTGATTGCTGTCAAC GCTAGGAGAAGGTGTGGGC (forward) and GCCACACCTTCTC CTAGCGTTGACAGCAATGCAGA (reverse); CD44 S291D, GTCTGCA TTGCTGTCAACGATAGGAGAAGGTGTGGCA (forward) and GCC CACACCTTCTCCTATCGTTGACAGCAATGCAGAC (reverse); CD44E, GACCTCAGATTCCAGAGAACAGTAGGAGAAGGTG (forward) and CACCTTCTCCTACTGTCTCTGGAATCTGAGGTC (reverse); NRG1 Δ ICD, CGGAATTCCCACCATGTCTGAGCGCAAAGAAGGCAGA (forward) and CTGAGTCTCGAGCTAATTCAGATCTTCTTCAGAAAT AAGCTTTTGTTCGCAGTAGGCCACCACA (reverse); NRG1 S289A, CTCCGGCAGAGCCTTCGGGCTGAACG (forward) and CGTTCAGC CCGAAGGCTCTGCCGGAG (reverse); NRG1 S336A/S338A, AGAGAA GTGGAGACCGCTTTTGCTACCAGTCACTACACTT (forward) and AAGTGTAGTGAAGTGGTAGCAAAAGCGGTCTCCACTTCTCT (reverse). All constructs were verified by sequencing.

Cell lines and transfections. NIH 3T3 fibroblasts were from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom). The human melanoma RPM-MC cells, negative for CD44, were kindly provided by Ivan Stamenkovic (University of Lausanne, Switzerland). Mouse embryonic fibroblasts (MEFs) with either *adam10* or *adam17* gene disruptions were kindly provided by Paul Saftig (University of Kiel, Germany) (37). The stable HEKNE wild type (WT), NRG1 mutant HEKNE S286A, and MEFE WT cell lines were created by retroviral infection with FLAG-NRG1 β 1a-EGFP (WT and S286A mutant). The pB-FLAG-NRG-EGFP retrovirus was used to infect HEK293T cells expressing the angiotensin I receptor (AT1R). All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). DNA and small interfering RNA (siRNA) transfections were performed in 6-well and 12-well plates (protease accessibility studies) or 10-cm and 15-cm plates (for biotinylation and immunoprecipitation [IP] experiments) using the liposomal transfection reagent Lipofectamine 2000.

Biotinylation of cell surface proteins. Before labeling, cells were washed three times with 1 $\times$ phosphate-buffered saline (PBS). For biotinylation cells were incubated with 5 ml of 0.5 mg/ml EZ-Link sulfo-NHS-LC-LC-biotin in 1 $\times$ PBS for 40 min at room temperature (RT) with gentle rocking. Afterwards, cells were washed once with 1 $\times$ PBS and incubated with 50 mM glycine in 1 $\times$ PBS for 15 min at 4°C with gentle rocking (to bind unreacted biotin). Finally, cells were washed once with 1 $\times$ PBS and incubated in fresh DMEM for 2 h in the absence or presence of tetradecanoyl phorbol acetate (TPA) induction. Batimastat was added to block ADAM activity (see Fig. 5B; see Fig. S1, S2A, and S6B in the supplemental material). After TPA induction cell culture supernatants were collected, and cells were lysed in IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl,

1% NP-40, 0.1% SDS, 2 mM EDTA) containing 1× complete protease inhibitor cocktail. Biotinylated proteins were precipitated with the use of streptavidin-Sepharose and analyzed by immunoblotting.

Inhibited cleavage conditions. Metalloprotease activity was blocked by culturing cells with a broad-spectrum hydroxamate-based metalloprotease inhibitor, 15 μ M GM-6001 or 5 μ M batimastat (BB94), 15 to 30 min prior to TPA or AngII stimulation. In addition, γ -secretase activity was blocked by 5 μ M DAPT or by 10 μ M compound E.

Precipitation of proteins by TCA-DOC. For detection of soluble CD44 ectodomain or soluble neuregulin (solCD44E or solNRG1E, respectively), cells were cultured in serum-free medium. Cell culture supernatants were precleared at 10,000 rpm for 10 min to pellet cell debris. Supernatant was mixed with a 1/100 volume of 2% deoxycholate (DOC), vortexed, and kept on ice for 30 min. Then, a 1/10 volume of 100% trichloroacetic acid (TCA) was added, and the samples were kept at 4°C overnight. The precipitate was recovered by centrifugation at 15,000 $\times$ g for 15 min, rinsed twice with acetone, and redissolved in radioimmuno-precipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS).

Co-IP from cell lysates. For coimmunoprecipitation (co-IP) experiments, transfected RPM-MC and NIH 3T3 cells were grown in 15-cm plates at low cell density (for co-IP of CD44 and ERM proteins) or at high cell density (for co-IP of CD44 and merlin). Cells were washed once in ice-cold 1× PBS and harvested in IP buffer containing 1× complete protease inhibitor cocktail (Roche, Basel, Switzerland). The following IP buffer was used for co-IP of CD44 with merlin or moesin: 10 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 0.5% NP-40, and 2 mM EDTA. After DNA was sheared, the lysate was precleared. Tagged CD44 was immunoprecipitated using 20 μ l of anti-FLAG M2 affinity gel beads (Sigma-Aldrich, St. Louis, MO). Endogenous merlin was immunoprecipitated using 30 μ l of Protein G Plus (Santa Cruz Biotechnology, Santa Cruz, CA) gel beads, preincubated with 2 μ g of merlin antibody. IP was performed with slow rotation at 4°C overnight. Isotype-matched control antibodies were used as negative controls to estimate the nonspecific binding of target proteins. Immunocomplexes were recovered by centrifugation, washed four times with cold IP buffer, and eluted with 2× Laemmli sample buffer.

Trigeminal ganglion explant assay. Embryonic tissue was obtained from C57BL/6 pregnant mice, which were sacrificed using CO₂ followed by cervical dislocation. Short hairpin RNA (shRNA) constructs in the retroviral GFP vector, pGFP-V-RS (Origene) expressing a unique 29-mer against protein kinase C delta (PKC δ) (NCBI GeneID 5580), and a non-effective 29-mer scrambled shRNA were electroporated *ex utero* at a concentration of 1 μ g/ μ l in mouse trigeminal ganglia (TG) at embryonic day 14.5 (E14.5) using three 100-ms pulses of 25 V at 1-s intervals as previously described (38). Electroporated TG were microdissected and cultured as neural explants in a collagen matrix at 37°C and 5% CO₂ for 3 days in the presence of nerve growth factor (NGF) to elicit neurite outgrowth (39). The explant cultures were cultured with or without soluble NRG1 (25 ng/ml) before being processed for fixation and immunostaining. In parallel, nonelectroporated TG explants were cultured in the presence of dimethyl sulfoxide (DMSO) or the broad-spectrum PKC inhibitor BIM1 with or without soluble NRG1 (25 ng/ml). All explants were processed for immunostaining as previously described (38) using the anti- β -tubulin III antibody, Tuj1, a neuron-specific marker, and the Schwann cell-specific cell marker S-100 β (N-15).

Trigeminal ganglion cocultures. Trigeminal nerve ganglia (TNG) isolated from mouse E14.5 embryos were dissociated for culture as previously described (39). Embryos were treated with 0.25 mg/ml of dispase I (D4818; Sigma) for 5 min prior to isolation, followed by TNG microdissection and incubation with 1 mg/ml of trypsin (T47-99; Sigma) for 10 to 12 min in a 37°C water bath. Dissected TNG were triturated with flamed glass pipettes, and a single-cell suspension of 10⁵ trigeminal ganglion cells was plated onto poly-D-lysine-coated (20 μ g/ml) and laminin-coated (10 μ g/ml) coverslips. Isolated TNG primary cultures were cocultured with organotypic mouse Schwann cells (postnatal day 1 to 2) for 7 days in

NGF-containing medium supplemented with 50 μ g/ml ascorbic acid (Sigma), which promotes myelinating conditions, with or without the broad-spectrum PKC inhibitor BIM1 (Calbiochem) before being processed for immunostaining with the anti- β -tubulin III antibody (Sigma), a neuron-specific tubulin marker, the Schwann cell-specific cell marker S-100 β (N-15) (Santa Cruz Biotechnology), and the basic myelin protein (BMP) antibody (Sternberger monoclonal antibody SMI; Covance).

Animals. All experiments involving animals were handled according to international guidelines on the ethical use of animals and animal experimentation and carried out in accordance with procedures approved by the local animal ethics committee (Thüringer Landesamt fuer Lebensmittelsicherheit und Verbraucherschutz).

Microscopy and immunofluorescence analysis. Fluorescent photographs were generated with a Zeiss Axio Imager ApoTome microscope, Zeiss LSM 710 confocal microscope system (Carl Zeiss AG, Jena, Germany), or a Nikon D-Eclipse C1 confocal laser scanning microscope (Tokyo, Japan) using identical acquisition parameters for each experiment using the confocal acquisition software Nikon EZ C1, version 3.90, Zen 2012 software, and the AxioVision Image Analysis software. In addition, for quantification analysis of TG explants, the neurite-occupied area was measured using the ImageJ plug-in Neurite-J, version 1.01, as described previously (40).

Immunofluorescence cell labeling. Cells were grown on coverslips placed at the bottom of 12-well plates. At 16 h after transfection, cells were washed in 1× PBS, and nonpermeabilized cells were incubated with either 10 μ g/ml Texas Red-WGA, Alexa Fluor 647-phalloidin, or rhodamine-phalloidin (Molecular Probes) in 1× PBS to label the plasma membrane (WGA) or the cytoskeleton (phalloidin conjugates). Cells were washed with 1× PBS and fixed with 4% paraformaldehyde (PFA) in cytoskeleton buffer (10 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA) at room temperature for 20 min. Afterwards, the cells were permeabilized using 0.1% Triton X-100 in 1× PBS (5 min at room temperature). Nonspecific epitopes were blocked by incubating the samples with 1% bovine serum albumin (BSA) in 1× PBS for 45 min. To label CD44 or NRG1, the samples were incubated with FLAG antibody at room temperature for 1 h. Afterwards, the samples were washed three times in 1× PBS and incubated with secondary anti-rabbit IgG conjugated to Alexa Fluor 488 for 1 h. For staining of the nucleus, 1 μ g/ml DAPI (Molecular Probes) was added together with the secondary antibody. After another three washing steps, the coverslips were mounted onto slides with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA). HEK293T, HEK293T S286A, and MEF2E WT cells were seeded at 8 $\times$ 10⁴ cells/well on BD BioCoat precoated (poly-D-lysine) 12-mm glass coverslips (Thermo Fisher Scientific) placed in 24-well plates. Cells were treated under either control (DMSO) conditions or under inhibited cleavage conditions for 30 min in the presence or absence of a stimulus (TPA or AngII) for 15 min as described above. After treatments, cells were washed in 1× PBS and fixed with warm 4% PFA for 20 min. Fixed cells were washed three times in 1× PBS before the cells were blocked in 5% PDT (1× PBS, 5% normal donkey serum, 1% BSA, 0.1% Triton X-100). Coverslips were processed for primary immunostaining overnight, followed by three subsequent washes in 1× PBS the next day and secondary antibody staining at room temperature in the dark for 2 h. After secondary antibody incubation, cells were washed three times with 1× PBS (10 min), and coverslips were mounted in Vectashield mounting medium containing DAPI (Vector Laboratories).

Statistical analysis. For statistical analysis, the intensity of bands from immunoblots was quantified using ImageJ and Image Lab (Bio-Rad, Hercules, CA) software. All values on histograms are reported as means $\pm$ standard deviations (SD). Statistical analyses of experiments were performed using an unpaired Student's two-tail *t* test of data analyzed from at least three independent experiments. Results are expressed as the means and standard errors of the means (SEM), with a *P* value of <0.05 as the significance level.

FIG 1 The ectodomains of NRG1 and CD44 determine the ADAM isoform shedding the molecules. (A) RPM-MC cells transfected with doubly tagged NRG1 (N-terminal FLAG and C-terminal c-myc tags) were grown at low cell density. Expression of ADAM10 (A10) or ADAM17 (A17) was downregulated by siRNA (to 3.8% and 1.6% as calculated from the blot by ImageJ). A nontargeting siRNA was used as a control (C). Cells were treated with 100 ng/ml TPA for 30 min. The released ectodomain was precipitated from culture supernatant by TCA prior to SDS-PAGE. Cleaved ectodomain (solNRG1E), NRG1fl, and NRG1ΔE were detected by FLAG and c-myc antibodies, respectively. The efficiency of siRNA knockdowns was monitored by detection of ADAM10 and ADAM17 proteins as indicated. Only ADAM17 knockdown and ADAM10/ADAM17 double knockdown reduced basal levels and induced release of solNRG1E and generation of NRG1ΔE. P, proform; M, mature form. (B and C) Experiments are identical to the experiment for panel A except that chimeric constructs were transfected with CD44E/NGR1(TM+ICD), carrying N-terminal FLAG and C-terminal GFP, and NRG1E/CD44(TM+ICD), carrying N-terminal FLAG and C-terminal c-myc. WB, Western blotting.

RESULTS

Substrate chimeras show that ADAM protease specificity is determined by the substrate's ectodomain irrespective of the identity of the ICD. One way to dissociate the roles of the intracellular domains (ICDs) and of the ectodomains in cleavage regulation is the use of chimeric constructs exchanging NRG1 and CD44 ectodomains or ICDs. This allowed us to first determine which domain is responsible for coupling the substrate to its “preferred” ADAM enzyme. Second, we tested whether the substrate-specific type of cleavage regulation, as determined in the WT molecule, is mediated by relevant intracellular signal-induced ICD modifications and whether these ICD modifications can regulate cleavage of a foreign ectodomain.

We have shown previously that CD44 is cleaved predominantly by ADAM10 (see reference 41 and Fig. 1B therein). A sim-

ilar experiment using ADAM downregulation by siRNA confirmed the major ADAM17 dependence of NRG1 proteolysis (Fig. 1A). N- and C-terminally double-epitope-tagged CD44 and NRG1 were transfected into RPM-MC cells transfected with either a control or an siRNA targeting ADAM10, ADAM17, or both. Cleavage was induced using TPA (a phorbol ester, diacylglycerol mimic, and PKC activator) and detected by measuring both the released ectodomain (solCD44E or solNRG1E) and the membrane-bound residual fragments (CD44ΔE or NRG1ΔE). To prevent loss of the membrane-bound cleavage product, we inhibited further processing by γ -secretase using DAPT. We focused exclusively on cleavage regulation after insertion of the substrates into the plasma membrane. To document this, we repeated several experiments after prior cell surface biotinylation and showed that biotinylated ectodomains are indeed released from the cell surface

in response to cleavage-regulatory stimuli (see Fig. S1 in the supplemental material). TPA induced NRG1 cleavage (Fig. 1A, compare lanes 1 and 5). siRNA-dependent downregulation of ADAM17 or of both ADAM10 and ADAM17 reduced NRG1 cleavage (Fig. 1A, lanes 6 and 8; P and M indicate precursor and mature forms, respectively). Downregulation of ADAM10, however, had no effect (Fig. 1A, lane 7).

To investigate whether the identity of the ICD affects the specific interaction between ectodomain and enzyme, we constructed chimeric molecules, as shown in the schematic of Fig. 1B. These chimeric molecules were transfected into RPM-MC cells and subjected to the same type of analysis as that used in the experiment shown in Fig. 1A. Downregulation of ADAM17 did not inhibit the cleavage of the chimeric construct carrying the CD44 ectodomain, while downregulation of ADAM10 did (Fig. 1B, compare lanes 7 and 8). Conversely, the NRG1 ectodomain was shed by ADAM17 irrespective of the presence of the CD44 ICD (Fig. 1C, lanes 7 and 8). Thus, the ectodomains carry the recognition sequence for the protease, and the ICD has no specific influence on this recognition. Based on our previous finding of substrate-specific cleavage regulation of NRG1 by ICD modification via PKC δ (5) and of CD44 by ICD interaction with the tumor suppressor merlin (4), we thus wondered whether this specific ICD regulation can be transferred to a foreign ectodomain.

Cleavage of WT NRG1 and CD44 is induced by different signaling pathways, and specificity of cleavage regulation can be transferred to a foreign ectodomain. The induction of ectodomain cleavage by RTKs or GPCRs often involves the activation of protein kinase C (PKC) family isoforms (5, 28, 29). We thus examined PKC-induced ectodomain cleavage of both CD44 and NRG1 using double-tagged transfected molecules (Flag-CD44-myc or Flag-NRG1-GFP) or their endogenous counterparts (as shown in Fig. S2A in the supplemental material, endogenous and transfected substrates are similarly cleaved). As shown in Fig. 1, cleavage products were either detected in the cell culture supernatant as soluble ectodomain (solCD44E and solNRG1E) or in the form of the residual C-terminal cleavage products (lacking the ectodomain; CD44 Δ E and NRG1 Δ E). Cleavage was induced by treating transfected RPM-MC cells with the phorbol ester TPA and with angiotensin II (AngII) in HEK cells carrying the angiotensin II type 1 receptor.

We first tested the effect of different protein kinase inhibitors on the cleavage of CD44 or NRG1 WT molecules. We found significant differences using PKC inhibitors. Gö6976 selectively inhibited the conventional PKC isoforms α and β , whereas bisindolylmaleimide 1 (BIM1), a broad-spectrum PKC inhibitor, additionally blocked the activities of the so-called novel PKC isoforms γ , δ , and ϵ but not the atypical PKC isoforms ζ and ι . Release of the CD44 ectodomain (solCD44E) or of the NRG1 C-terminal cleavage products in response to TPA, or, in the case of NRG1, also in response to AngII stimulation, could be inhibited by BIM1 (Fig. 2A, B, and E). In contrast, CD44 cleavage was blocked by Gö6976 only after TPA treatment (Fig. 2C), while NRG1 cleavage was not by either TPA (Fig. 2D) or AngII treatment (Fig. 2E; see also Fig. S2B in the supplemental material), suggesting the involvement of PKC α/β isoforms in CD44 but not in NRG1 cleavage. For clarity, quantification of results for CD44 and NRG1 are shown underneath the immunoblots (Fig. 2A to D) or in bar graphs (Fig. 2A/C' and E', showing the means of three experiments).

We also tested a number of other inhibitors for their effects on

the cleavage of the WT molecules; most of these inhibitors did not affect the substrates already inserted into the plasma membrane (data not shown). One inhibitor, however, was informative: okadaic acid, an inhibitor of protein phosphatases 1 and 2 (PP1/2), which account for about 90% of phosphatase activity in eukaryotic cells (42). While TPA-induced CD44 cleavage was blocked by okadaic acid (Fig. 2F), NRG1 cleavage was totally resistant to inhibition (Fig. 2G). In summary, these results suggest that induced CD44 cleavage involved activation of one of the classical PKC isoforms (PKC α or PKC β) and a protein phosphatase, while induced NRG1 cleavage was addressed by one of the novel PKC isoforms and did not involve a phosphatase.

Next, we tested in chimeras whether these differences in cleavage behavior of CD44 and NRG1 are mediated by the relevant ICD modifications and whether this behavior is transferrable to a foreign ectodomain. Expectedly, this is the case: in NRG1 or CD44 ectodomain/ICD chimeras, PKC inhibitor sensitivity of the chimera is determined by the ICD (Fig. 2H). Cleavage of the chimera CD44E/NRG1(TM+ICD) was inhibited by BIM1, but not by Gö6976, as with the NRG1 WT molecule (Fig. 2D). The reverse chimera showed inhibition by both BIM1 and Gö6976 (data not shown) (see also below), which is characteristic of the CD44 WT molecule (Fig. 2C). In summary, the ICDs determine substrate-specific cleavage behavior in response to specific signaling pathways.

This specificity is also maintained in the case of CD44 cleavage regulation by merlin. While CD44 cleavage is inhibited by the tumor suppressor merlin (NF2) (4), neuregulin release is resistant to merlin inhibition. TPA-induced CD44 cleavage was reduced by the constitutively active mutant of merlin, NF2 S518A (Fig. 3A, compare second and fifth lanes) but not by the inactive mutant NF2 S518D (Fig. 3A, third and sixth lanes). Neuregulin release was not affected by either merlin mutant (Fig. 3B). A quantification of these results and a comparison of data for CD44 and NRG1 are shown in the bar graph (Fig. 3A/B'). This specificity of regulation is explained by the fact that the NRG1 ICD lacks a merlin binding domain. However, in an NRG1E/CD44(TM+ICD) chimera, release of the NRG1 ectodomain becomes dependent on merlin, which is revealed when a mutant CD44 ICD is fused to the NRG1 ectodomain which is unable to bind merlin (lysine-arginine-rich mutant [KR-MT]; further introduced and studied below) (Fig. 3C, compare bar graphs of WT and KR-MT).

We conclude that substrate ICDs determine the type of mechanism or modification that controls cleavage and that they can impose this regulation onto a foreign ectodomain. The ectodomain, however, determines which specific ADAM cleaves the molecule. As a next step, we attempted to dissect the relevant regulatory ICD modifications further, starting with CD44 and followed by NRG1.

Role of the CD44 ICD in cleavage regulation. To identify the specific ICD structural determinants and modifications relevant for induced shedding, we first tested CD44 ICD deletion mutants (carrying only the N-terminal FLAG tag) in cleavage assays. The truncated CD44 constructs (Fig. 4A, schematic) were properly expressed on the cell surface (see Fig. S3 in the supplemental material). Immunoblots of four independent experiments were quantified, and results are shown as bar graphs (for additional examples of the quantified immunoblots of Fig. 4, see Fig. S4 in the supplemental material). Surprisingly, while cleavage of CD44 WT required TPA stimulation (Fig. 4A), the ICD-less CD44

FIG 2 Different signaling pathways are involved in the induced cleavage of CD44 and NRG1. (A) TPA-induced CD44 cleavage is inhibited by the broad-spectrum PKC inhibitor BIM1. (B) TPA-induced NRG1 cleavage is inhibited by BIM1. (C) TPA-induced CD44 cleavage is inhibited by the PKC α / β -specific inhibitor G66976. (D) TPA-induced NRG1 cleavage cannot be blocked by the PKC α / β -specific inhibitor G66976. (E) Angiotensin II (AngII)-induced NRG1 cleavage is inhibited by BIM1 but not by the PKC α / β -specific inhibitor G66976. (F) TPA-induced CD44 cleavage is inhibited by increasing concentrations of the protein phosphatase 1/2 inhibitor okadaic acid. (G) TPA-induced NRG1 cleavage is not blocked by okadaic acid treatment. (H) The NRG1 ICD in the chimeric substrate determines the spectrum of inhibition. Cleavage of transfected FLAG/myc-tagged standard isoform of wild-type CD44 was studied in CD44-deficient RPM-MC cells. Cleavage of stably expressed FLAG/GFP-tagged NRG1 was studied in HEK293T cells expressing angiotensin II type 1 receptor. We measured cleavage in different cell lines (CD44-negative RPM-MC melanoma cells, NIH 3T3 fibroblasts, and HEK293T cells) but found no significant cell-type-specific differences. To activate PKC, we used either phorbol ester (100 ng/ml TPA; treatment of 2 h for CD44 and 30 min for NRG1), a mimic of diacylglycerol that activates most PKC isoforms, or we used activation of the angiotensin II (AngII) receptor, a GPCR coupled to PKC activation, by angiotensin (100 ng/ml AngII; treatment of 30 min for NRG1). γ -Secretase inhibitor DAPT (5 μ M) was added in all experiments to avoid subsequent further C-terminal proteolysis and to exclude confounding cleavage events. Cleaved soluble CD44 ectodomain (solCD44E) and cleaved soluble neuregulin (solNRG1E) were detected by anti-FLAG immunoblotting of TCA-DCC-precipitated cell culture supernatants. CD44 Δ E and NRG1 Δ E (membrane-bound cleavage products lacking the ectodomain) as well as the full-length molecules (CD44fl and NRG1fl) were detected by c-Myc or GFP immunoblotting in cell lysates, except in the case of the experiment shown in panel E (C-terminal NRG1 antibody). Inhibitors were added at the concentrations indicated. G66976 (Gö) selectively inhibits the conventional PKC isoforms α and β , whereas bisindolylmaleimide 1 (BIM1), a broad-spectrum PKC inhibitor, additionally blocks the activities of the so-called novel PKC isoforms γ , δ , and ϵ . The bar graphs in panels A/C' and E' show mean values of relative levels of NRG1 Δ E and solCD44E $\pm$ SD from three independent experiments (repeats of experiments shown in panels A, C, and E). The P values for CD44 are 0.011389 (BIM1) and 0.027016 (G66976). The P values for NRG1 are 0.023442 (BIM1) and 0.031415 (G66976). In panel H one of the chimeric constructs shown in the schematic was transfected into RPM-MC cells and treated as described for panels A and B.

(CD44 Δ ICD) was constitutively cleaved in the absence of TPA, and cleavage could not be further enhanced by TPA (Fig. 4A). Constitutive cleavage was nevertheless the result of metalloprotease activity and was inhibited by batimastat (41; also data not shown). These results indicate that the ICD of CD44 represses its

ectodomain cleavage, a block that—taking the PKC inhibitor data (Fig. 2) into account—might need to be overcome by a PKC-induced posttranslational modification of the CD44 WT ICD.

To explore which part of the ICD conferred inducibility of CD44 cleavage, we tested several ICD truncations, as shown in the

FIG 3 Inhibition of CD44 but not NRG1 ectodomain cleavage by merlin (NF2). Overexpression of a constitutively active merlin mutant inhibits CD44 (A) but not NRG1 (B) cleavage. RPM-MC cells were cotransfected with CD44 WT or NRG1 WT and merlin (NF2) S518A (active) or S518D (inactive) and kept at low cell density. Cleavage was detected as described in the legends of Fig. 1 and 2. (A/B') Quantification of the data in panels A and B. (C) Effect of merlin on the cleavage of a NRG1E/CD44(TM+ICD) chimera. Mutation of the merlin binding domain KR (KR-MT) blocks TPA-induced release of the NRG1 ectodomain in the chimera. Further, the effect of serine mutations at S291 (adjacent to the KR motif) on the cleavage of the chimera is examined (the relevance of S291 is fully introduced and detailed in Fig. 4 and the corresponding text).

schematic in Fig. 4A. Retaining the first serine following the transmembrane (TM) region (Fig. 4A, ΔafterS291) did not rescue inducibility, and cleavage remained constitutive (Fig. 4A). Retention of the adjacent basic amino acid stretch (which we call lysine-arginine-rich [KR] motif; the binding site for the cleavage-regulatory tumor suppressor merlin [NF2]) (43) up to N304 (ΔafterKR) reestablished TPA-induced cleavage (Fig. 4A). This minimal cleavage-regulatory ICD sequence (NSRRRCGQKKLVIN) (Fig. 4A) carries several amino acids that could be subject to posttranslational modification, such as serine phosphorylation/dephosphorylation, R or K acetylation, or cysteine oxidation. The amino acid sequence surrounding serine 291 (S291; which partially overlaps the minimal cleavage-regulatory KR sequence) does not correspond to the known PKC consensus phosphorylation site (serine residues surrounded by Arg or Lys at the -2 and +2 positions and a hydrophobic residue at the +1 position); however, PKC may act upstream of any of the putative CD44 ICD modifications. We mutated possibly modified amino acid positions such that no modifications were possible (Fig. 4B, box at top). Replacing basic

amino acids of the KR motif by alanines in full-length CD44 WT (KR-MT) severely reduced TPA-induced cleavage, as evidenced by negligible release of CD44 ectodomain (solCD44E) or C-terminal cleavage products (CD44ΔE) from the mutant (Fig. 4B, KR-MT). Surprisingly, this mutation did not cause constitutive cleavage, as may have been expected based on results with the CD44 ICD deletion mutants (Fig. 4A), indicating the existence of an additional regulatory event. This additional regulatory event might be represented by modification of serine S291: exchange of the serine S at position 291 for alanine (S291A) generated a constitutively cleaved molecule that showed release of the CD44 C-terminal cleavage product (CD44ΔE) even in the absence of TPA stimulation; TPA had only a slight further effect on cleavage, but this was not significant compared to the effect of TPA on WT CD44 (Fig. 4C). As alluded to in the discussion, the mutant S291A permits the cleavage-promoting binding of ERM proteins, which themselves are activated by TPA-induced phosphorylation; this might explain the small cleavage-enhancing effect of TPA in this mutant. An acidic substitution, S291D, mimicking serine phos-

FIG 4 Roles of the CD44 ICD in cleavage regulation. (A) Analysis of minimal sequence requirements of the ICD for regulated CD44 cleavage. (B) Mutation of the ERM binding motif in CD44 (KR-MT) inhibits cleavage. (C) Effect of S291 mutations on CD44 cleavage. (D) Coprecipitation of CD44 ICD mutants with merlin from high-cell-density cultures. (E) Coprecipitation of the ERM protein moesin with CD44 from low-cell-density cultures. (F) CD44 S291 phosphorylation by incorporation of ^{32}P orthophosphate. Tagged mutant CD44 constructs as indicated in panel A, the box in panel B, and the schematic in Fig. 2H were transfected into RPM-MC cells, and their cleavage induction by TPA was analyzed as described in the legend of Fig. 2. For co-IP of moesin RPM-MC cells were grown at low cell density to permit moesin activation (E). For merlin co-IP we used NIH 3T3 cells that express higher levels of merlin. To activate merlin, cells were grown to high cell density (D). Immunoblot bands were quantified using ImageJ and Image Lab. The bar graphs show mean values of relative levels $\pm$ SD from three independent experiments. Statistical significance was determined by Student's *t* tests with a *P* value of <0.05 as statistically significant. ns, not significant ($P = 0.120170$); *, $P = 0.022557$; **, $P = 0.006170$; ****, $P < 0.0001$. (F) The CD44 WT or CD44 ICD construct with deletion after S291 (CD44 Δ afterS291) was transfected into cells grown in the presence of ^{32}P orthophosphate. CD44 was precipitated with an anti-FLAG N-terminal tag antibody, and the precipitated immune complex was extensively washed prior to SDS-PAGE. Shown are representative autoradiographs of ^{32}P orthophosphate incorporation into CD44 WT and CD44 Δ afterS291; no significant dephosphorylation was detectable after TPA treatment. The leftmost panel shows an anti-FLAG immunoblot of the immunoprecipitate. V, vector.

phorylation, behaved in the same way as CD44 KR-MT mutant in that TPA was unable to induce its cleavage (Fig. 4C). Cleavage responses of CD44 WT and CD44 KR-MT are shown for comparison in Fig. 4C. Because the CD44 ICD carries other serines in addition to S291, we mutated these residues as well but did not find any effect on CD44 cleavage (see Fig. S5 in the supplemental material). Introduction of the cleavage-inhibitory S291D mutation into NRG1/CD44(TM+ICD) chimeras inhibited NRG1 ectodomain release in the chimera at a level similar to that of the CD44 WT (Fig. 3C). However, in contrast to the CD44 WT molecule, the S291A mutant did not cause constitutive cleavage of the NRG1 ectodomain in the chimera, but cleavage required TPA (Fig. 3C). We do not have a clear explanation for this inconsistency.

Since the KR motif has previously been identified as a binding site for proteins of the ERM (ezrin-radixin-moesin) family as well as for their counterplayer, the tumor suppressor protein merlin (12), we hypothesized that the immediately adjacent S291 phosphorylation might regulate ERM/merlin binding to the KR do-

main. ERM proteins and merlin are themselves regulated by phosphorylation but in opposite directions: merlin is active in the dephosphorylated state (see also the effect of the mutants in the experiment shown in Fig. 3), a condition met by high cell density, while ERM proteins are active when phosphorylated at low cell density during cellular proliferation. Active ERM proteins promote CD44 cleavage while active merlin inhibits it (4). As expected, the CD44 KR-MT mutant could not be coprecipitated either with ERM proteins (from lysates of proliferating cells) (data not shown) or with active merlin under high-cell-density conditions (Fig. 4D). More importantly, however, modification of serine S291 alone disturbed the interaction with ERMs, exemplified for moesin here: while the constitutively cleaved mutant protein CD44 S291A coprecipitated the activated ERM protein moesin (at low cell density), the uncleavable CD44 S291D mutant could not bind to moesin (Fig. 4E). CD44 S291D mimics phosphorylation of S291, a condition that would physiologically occur at high cell density when dephosphorylation of CD44 S291 is blocked and merlin is activated. Under these conditions, merlin binding to

putative CD44-phospho-S291 apparently occurs and mediates contact inhibition (12). Merlin, accordingly, can be coprecipitated with CD44 S291D from lysates of cells at high cell density (Fig. 4D). It is under these conditions that merlin blocks the cleavage of CD44. Overexpression of a constitutively active mutant of merlin, NF2 S518A, which does not require activating dephosphorylation, inhibited CD44 cleavage also at low cell density (Fig. 3A), indicating that merlin can interact with dephosphorylated CD44 S291 (Fig. 4D). Therefore, merlin can also be coprecipitated with CD44 S291A, which mimics the dephosphorylated state (Fig. 4D).

Taken together, these results suggest that serine phosphorylation at S291 might need to be removed for a cleavage-relevant interaction to occur and thus allow CD44 cleavage to proceed. Consistent with this, short-term incubations of cells with the phosphatase inhibitor okadaic acid completely abrogated CD44 cleavage (Fig. 2F). Because the CD44 ICD carries several serines, all of which are subject to phosphorylation/dephosphorylation, direct detection of S291 dephosphorylation proved difficult. In particular, our own attempts to detect induced dephosphorylation of a tryptic peptide comprising S291 by mass spectrometry have not been successful; this was likely due to the very close proximity of S291 to the membrane, causing reduced detectability of the tryptic peptide in mass spectrometry. However, by ^{32}P incorporation we were able to show that the CD44 WT and a CD44 mutant with an ICD truncation right after S291 (Fig. 4A, Δ afterS291) were phosphorylated *in vitro* (Fig. 4F). There was, however, no convincing evidence for TPA-induced dephosphorylation of CD44 Δ afterS291, possibly because the KR motif had been removed. Therefore, our tentative conclusion is that PKC-dependent dephosphorylation of S291 permits the binding of regulatory ERM proteins to the KR motif, which allows ADAM cleavage of CD44 to proceed.

Role of the NRG1 ICD in cleavage regulation. Analysis of the NRG1 ICD deletion mutant (Fig. 5B, NRG1 Δ ICD) yielded a totally different result from that of CD44 ICD deletion. While TPA induced a reduction of the full-length NRG1 (NRG1fl) WT (NRG1-EGFP; C-terminal GFP tag) and an increase of its cleavage products solNRG1E and NRG1 Δ E over a period of 5 to 120 min (Fig. 2B, D, and G and 5A), NRG1 Δ ICD was resistant to induced cleavage (Fig. 5A to C). Consequently, while cleavage of NRG1fl WT could be inhibited by batimastat or BIM1, these inhibitors had no effect on NRG1 Δ ICD (Fig. 5B). However, in contrast to the CD44 ICD deletion mutant, the NRG1 ICD deletion mutant showed a trafficking defect and was not properly inserted into the plasma membrane (see Fig. S6 in the supplemental material). Thus, in contrast to findings for the ICD of CD44, the NRG1 ICD is essential for the transport of NRG1 to the surface, and removal leads to reduced surface expression and reduced cleavage.

In contrast to complete ICD deletion, point mutants of several serines in the NRG1 ICD did not affect trafficking to the cell surface and could be examined for their effect on cleavage (Fig. 5D; see also Fig. S6 in the supplemental material). Compared to results in the NRG1 WT, production of the NRG1 ectodomain (solNRG1E) or C-terminal cleavage product (NRG1 Δ E) was delayed by mutation of S286 (NRG1 S286A) (Fig. 5F; compare with WT in E) or further delayed when this mutation was combined with two other serine mutations (NRG1 S286A/S336A/S338A) (Fig. 5G). The double mutant S336A/S338A showed an intermediate phenotype compared to that of the NRG1 WT or the com-

bination of all three mutations (compare Fig. 5E through H). However, mutation of S289 alone exerted a strong effect on cleavage on its own: cleavage of mutant NRG1 S289A was reduced, as shown by an almost complete lack of induced production of C-terminal cleavage products (NRG1 Δ E) and little release of soluble ectodomain (solNRG1) (Fig. 5I). These results suggest that the NRG1 ICD is modified by several serine phosphorylations, the combination of which activates its cleavage. Regulation may be affected by more than one protein kinase. We previously identified PKC δ (also activated by TPA) as one of the involved kinases responsible for phosphorylation of NRG1 at S286. This kinase is also, at least in part, responsible for regulation of NRG1 cleavage, as downregulation of PKC δ blocked NRG1 release (5). Using a phospho-specific antibody detecting the phosphorylated PKC consensus phosphorylation site, we could demonstrate that S286 is indeed modified within cells, that angiotensin II- or TPA-induced phosphorylation of S286 is absent, and that cleavage is inhibited when PKC δ is downregulated (5). These data prompted us to examine the role of the ICD modifications *in vivo*. We already showed that cleavage of CD44 is relevant for cellular migration (4). We next tested the potential physiological role of ICD modification in neuregulin release *in vivo*.

Downregulation of PKC δ inhibits neurite outgrowth and myelin production by Schwann cells in trigeminal nerve ganglia. Neuregulin release is of particular importance for neurite outgrowth as well as for migration and association of Schwann cells with axons and, thereby, myelination of such outgrowing neurites (44–46). NRG1 type I and NRG1 type III catalyze two subsequent steps: neurite outgrowth followed by myelination. To prove that the above reported regulation of NRG1 is physiologically relevant for the organism, we blocked the cleavage of NRG1 by shRNA downregulation of PKC δ in trigeminal nerve ganglia (TNG) and analyzed their neurite outgrowth and related Schwann cell migration and neurite association in *ex vivo* TNG explants. Downregulation of PKC δ would be expected to inhibit cleavage of endogenous NRG1 type I by blocking PKC δ -mediated phosphorylation at NRG1 S286 and thereby reducing neurite outgrowth, the association and migration of Schwann cells along outgrowing axons, and subsequently the reduction in NRG1 type III-dependent myelination. Different PKC δ shRNAs were tested for their knockdown ability by PKC δ immunoblotting in HEK293T cells. We chose one shRNA that stably eliminated PKC δ and that we had previously tested in the same cell line (5) for *ex vivo* experiments. Control and PKC δ shRNA-expressing plasmids were electroporated into TNG explants (Fig. 6B), and neurite outgrowth was monitored over time (Fig. 6C shows an exemplary micrograph of control-treated TNG labeled with Tuj-1, a neuronal marker, and DAPI for cell nuclei). TNG-expressing PKC δ shRNA showed significantly reduced neurite outgrowth compared to a control shRNA expressing TNG. This effect could be partially rescued by addition of soluble NRG1 (solNRG1E) to the explant culture (Fig. 6C; compare the neuron-specific Tuj-1 marker in all micrographs; bar graphs represent quantification of results from three different experiments performed in biological duplicate). Schwann cell association and migration along outgrowing axons were reduced as well and could also be partially rescued with solNRG1E (Fig. 6C, compare the S-100 β Schwann cell markers across micrographs). Similar reductions in neurite outgrowth and Schwann cell migration were also obtained by treating explant cultures with the PKC inhibitor BIM1 (inhibits classical and novel PKC isoforms, in-

FIG 5 Role of the NRG1 ICD in cleavage regulation. (A to C) Analysis of NRG1 ICD complete deletion mutants on NRG1 cleavage. CK (protein schematic in panel B), cysteine and lysine residues just below the transmembrane domain of NRG1. (D) Schematic of the NRG1 ICD serine mutants generated. (E to I) Analysis of the effect of NRG1 ICD serine mutations on NRG1 cleavage. N-terminally FLAG-tagged NRG1 WT or a corresponding construct lacking the ICD sequences was tagged by GFP at the C terminus (A to C) and introduced into HEK293T cells. Serine mutants (schematic in panel D; E to I) were generated by site-directed mutagenesis using N-terminally FLAG- and C-terminally myc-tagged NRG1 WT as a template. Cleavage products were detected as described in the legend of Fig. 2B. Batimastat (5 μ M; metalloprotease inhibitor) or 10 μ M BIM1 was added where indicated.

cluding PKC δ) (Fig. 7A). BIM1 treatment consequently reduced myelination of trigeminal neurons, as shown by reduced myelin basic protein (MBP) staining in dissociated TNG explants cocultured with organotypic mouse Schwann cells (Fig. 7B, compare MBP panels).

DISCUSSION

Our results show that two ADAM metalloprotease substrates, the precursor of the ErbB3/4 ligand growth factor neuregulin and the cell migration modulator CD44, can be specifically selected for cleavage by substrate-specific ICD modification. This mechanism complements regulatory mechanisms at the protease level.

The regulation we observed is mediated by members of the PKC class of kinases that are activated by numerous surface receptors. In the case of CD44 cleavage regulation, PP1/2 is critical for

cleavage induction. Putative dephosphorylation at CD44 S291 (based on mutational analysis and phosphate labeling) (Fig. 4) permits binding of the cleavage-regulatory ERM proteins. In addition to targeting the CD44 ICD for dephosphorylation to induce cleavage, TPA stimulation of cells also causes an activating phosphorylation of the ERM proteins. Extensive activation and binding of ERM proteins to CD44 S291A might explain why cleavage of this constitutively cleaved mutant can still be slightly enhanced by TPA (Fig. 4C). We thus propose that TPA activates a PKC-dependent PP1/2 serine phosphatase that acts on a putative phospho-S291 in the CD44 ICD. PP1/2 phosphatases are indeed regulated by endogenous PKC-activated inhibitors (42); as an example, we have shown that the endogenous negative PP1 regulator PPP1R14D is required for ADAM17-mediated EGF ligand cleavage but not for that of NRG1 (5), consistent with the lack of

FIG 6 Inhibition of endogenous NRG1 type 1 cleavage by PKC δ knockdown reduces neurite outgrowth and Schwann cell association of trigeminal nerve ganglion (TNG) explants. (A) Schematic diagram of the trigeminal explant assay. (B) Normal neurite outgrowth as detected in trigeminal explant assays. DIV3, 3 days *in vitro*. (C) Isolated TNG from mouse embryos were electroporated with a GFP retroviral vector (pGFP-V-RS) expressing either control shRNA or PKC δ shRNA. Neurite outgrowth was monitored with the neuronal marker Tuj-1 and Schwann cell association with the S-100 β marker. DAPI was used to visualize nuclei. Shown are representative micrographs of two independent experiments of 5 to 10 biological replicates each. Images were quantified using ImageJ and Image Lab. Scr, scrambled; mE14.5, mouse E14.5 embryo.

effect of okadaic acid-dependent phosphatase inhibition on NRG1 cleavage (Fig. 2G).

We published a previous report (5) and now present additional and plausible evidence that, in contrast to CD44, induced NRG1 ectodomain release depends on phosphorylations of several NRG1 ICD serines (Fig. 5). NRG1 cleavage is independent of ERM proteins or merlin (Fig. 3) (4). Because delays in protein trafficking to the cell surface would mimic reduced cleavage, we applied cell surface biotinylation very shortly before cleavage induction to ensure that all WT molecules and mutants (except for the NRG1 ICD deletion) were properly located on the plasma membrane and that we were therefore studying the regulatory steps of NRG1 (or CD44) cleavage at the cell surface (for an example, see Fig. S1 in the supplemental material). Surface expression was also ascertained by confocal microscopy in control experiments (see Fig.

S6). Complete ICD deletion in NRG1 led to retention of the molecule within the cell and therefore to a block in its ectodomain cleavage, which normally occurs at the cell surface. Our *in vitro* results on NRG1 Δ ICD trafficking and the observation that NRG1 ICD deletion in mice recapitulates the phenotype caused by complete knockout of NRG1 in mice suggest that regulation of trafficking of NRG1 by its ICD could be important for cleavage regulation of NRG1 *in vivo* (47, 48). Similar to our NRG1 β 1a deletion mutant, a NRG1 α 2c C-terminal deletion mutant was also resistant to cleavage under basal conditions (49). However, point mutations of serines in NRG1 did not appear to reduce trafficking of NRG1 to the cell surface although they affected NRG1 cleavage (Fig. 5; see also Fig. S6 in the supplemental material). This suggests that phosphorylation/dephosphorylation of serines in the NRG1 ICD indeed represents a cleavage-regulatory step and affects

FIG 7 Disruption of endogenous NRG1 type 1 cleavage by PKC inhibition (BIM1) decreases neurite outgrowth, Schwann cell migration, and myelination of trigeminal nerve ganglion (TNG) neurons. (A) PKC inhibition with BIM1 reduces neurite outgrowth and Schwann cell migration in mouse embryonic day (E) 14.5 TNG explants after 3 days *in vitro* (DIV); the inhibition can be partially rescued by the addition of soluble NRG1 (bottom panel). Scale bar, 100 μ m. (B) Dissociated TNG cocultured with organotypic mouse Schwann cells show reduced myelination in the presence of BIM1 (compare myelin basic protein [MBP] panels). TNG were isolated from mouse E14.5 embryos as described in the legend of Fig. 6, dissociated, and cultured with postnatal (day 1 to 2) mouse Schwann cells under myelinating conditions (50 μ g/ml ascorbic acid) as previously described (38). Neurite outgrowth was monitored with the anti- β -tubulin III Tuj-1 antibody, a neuron-specific tubulin marker; Schwann cell migration along neurites was detected using the specific Schwann cell marker S-100 β , and a myelin basic protein (MBP) antibody was used to detect myelination. Shown are representative micrographs. Scale bar, 50 μ m.

cleavage of the molecule on the cell surface. Consistent with our findings for mouse NRG1, cleavage of chicken NRG1 in neuronal cells is also regulated by PKC δ and phosphorylated ICD serine residues (50).

Published work by other researchers further supports the concept of cleavage-regulatory ICD modifications: unlike CD44, the ICDs of L-selectin and angiotensin-converting enzyme (ACE) are constitutively bound to calmodulin, which represses their cleavage, and calmodulin kinase inhibition or stimulation by TPA activates processing (51, 52). Binding of ERM proteins to the L-selectin ICD was required for its PKC-induced cleavage (53). A phospho-mimicking mutant of L-selectin S367 (close to its ERM binding motif) enhanced cleavage, suggesting that the S367 phosphorylation state regulates ERM interaction (53), very similar to our findings for the phosphorylation state of S291 in CD44. Also, ERM binding was required for induced shedding of amyloid precursor proteins (APPs) (54). Of note, cleavage of one isoform of angiotensin-converting enzyme in endothelial cells depends on dephosphorylation of an ICD phosphoserine (55), again similar to our findings for CD44.

However, not all substrates require their ICDs for cleavage regulation and not all induced ICD phosphorylation events regulate cleavage (e.g., interleukin-6 [IL-6] receptor) [56], tumor necrosis factor alpha [TNF- α] receptor II [57], and heparin-binding EGF [HB-EGF] [58]). Because ADAM17 and ADAM10 also do not require their ICDs for induced cleavage (30, 59–61), these findings suggest the existence of other regulatory proteins that could also receive ICD signaling input. As examples, inactive rhomboid pro-

teins (iRhoms) (62) and annexins (63, 64) interact with ADAM17 and/or substrates on the cell surface and regulate the cleavage of select ADAM17 substrates. The mechanisms involved in this regulation still have to be elucidated.

Some of our results need further explanation and suggest additional uncovered regulatory components. In one early study CD44 S291A was reported to inhibit cellular migration (65), while we found that it enhances CD44 cleavage, a feature that is required for migration in our hands (4). The reason for this discrepancy has yet to be uncovered. Also unexplained is why the CD44 KR mutant (mutated cleavage-regulatory ERM/merlin binding domain) is not constitutively cleaved in the same way as the CD44 ICD deletion mutant (Fig. 4B and C). Why did the CD44 S291A mutant ICD only very mildly elevate spontaneous cleavage of the NRG1/CD44 chimera (and required TPA for cleavage) compared to its effect in the CD44 full-length parent molecule (compare Fig. 4C and 3C)?

In summary, regulation of ectodomain cleavage through ICD modification explains how substrate specificity is achieved, which is absolutely necessary for the controlled release of numerous essential regulatory molecules. Our findings provide the exciting opportunity to devise experiments that will address substrate-level cleavage regulation in detail and determine its relation to metalloprotease level regulation. Knowledge of the signaling pathways that lead to specific ICD modifications on substrates might allow the selective inhibition of substrate cleavage therapeutically without inhibiting the metalloprotease and thus without affecting the cleavage of many substrates at the same time, a major reason

for the failure of early unspecific metalloprotease inhibitors in the clinic (66, 67).

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REFERENCES

- Dreymueller D, Pruessmeyer J, Groth E, Ludwig A. 2011. The role of ADAM-mediated shedding in vascular biology. *Eur J Cell Biol* 91:472–485. <http://dx.doi.org/10.1016/j.ejcb.2011.09.003>.
- Hartmann M, Herrlich A, Herrlich P. 2013. Who decides when to cleave an ectodomain? *Trends Biochem Sci* 38:111–120. <http://dx.doi.org/10.1016/j.tibs.2012.12.002>.
- Montes de Oca-B P. 2010. Ectodomain shedding and regulated intracellular proteolysis in the central nervous system. *Cent Nerv Syst Agents Med Chem* 10:337–359. <http://dx.doi.org/10.2174/187152410793429700>.
- Hartmann M, Parra LM, Ruschel A, Schubert S, Li Y, Morrison H, Herrlich A, Herrlich P. 2015. Tumor suppressor NF2 blocks cellular migration by inhibiting ectodomain cleavage of CD44. *Mol Cancer Res* 13:879–890. <http://dx.doi.org/10.1158/1541-7786.MCR-15-0020-T>.
- Dang M, Armbruster N, Miller MA, Cermenio E, Hartmann M, Bell GW, Root DE, Lauffenburger DA, Lodish HF, Herrlich A. 2013. Regulated ADAM17-dependent EGF family ligand release by substrate-selecting signaling pathways. *Proc Natl Acad Sci U S A* 110:9776–9781. <http://dx.doi.org/10.1073/pnas.1307478110>.
- Fleck D, van Bebbler F, Colombo A, Galante C, Schwenk BM, Rabe L, Hampel H, Novak B, Kremmer E, Tahirovic S, Edbauer D, Lichtenthaler SF, Schmid B, Willem M, Haass C. 2013. Dual cleavage of neuregulin 1 type III by BACE1 and ADAM17 liberates its EGF-like domain and allows paracrine signaling. *J Neurosci* 33:7856–7869. <http://dx.doi.org/10.1523/JNEUROSCI.3372-12.2013>.
- Newbern J, Birchmeier C. 2010. Nrg1/ErbB signaling networks in Schwann cell development and myelination. *Semin Cell Dev Biol* 21:922–928. <http://dx.doi.org/10.1016/j.semcdb.2010.08.008>.
- Nave KA, Salzer JL. 2006. Axonal regulation of myelination by neuregulin 1. *Curr Opin Neurobiol* 16:492–500. <http://dx.doi.org/10.1016/j.conb.2006.08.008>.
- Liu FF, Stone JR, Schuldt AJ, Okoshi K, Okoshi MP, Nakayama M, Ho KK, Manning WJ, Marchionni MA, Lorell BH, Morgan JP, Yan X. 2005. Heterozygous knockout of the neuregulin-1 gene in mice exacerbates doxorubicin-induced heart failure. *Am J Physiol Heart Circ Physiol* 289: H660–H666. <http://dx.doi.org/10.1152/ajpheart.00268.2005>.
- Jin H, Sperka T, Herrlich P, Morrison H. 2006. Tumorigenic transformation by CPI-17 through inhibition of a merlin phosphatase. *Nature* 442:576–579. <http://dx.doi.org/10.1038/nature04856>.
- Tian X, Azpurua J, Hine C, Vaidya A, Myakishev-Rempel M, Ablueva J, Mao Z, Nevo E, Gorbunova V, Seluanov A. 2013. High-molecular-mass hyaluronan mediates the cancer resistance of the naked mole rat. *Nature* 499:346–349. <http://dx.doi.org/10.1038/nature12234>.
- Morrison H, Sherman LS, Legg J, Banine F, Isacke C, Haipiek CA, Gutmann DH, Ponta H, Herrlich P. 2001. The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. *Genes Dev* 15:968–980. <http://dx.doi.org/10.1101/gad.189601>.
- Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. 2003. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 100:3983–3988. <http://dx.doi.org/10.1073/pnas.0530291100>.
- Patrawala L, Calhoun T, Schneider-Broussard R, Li H, Bhatia B, Tang S, Reilly JG, Chandra D, Zhou J, Claypool K, Coghlan L, Tang DG. 2006. Highly purified CD44⁺ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene* 25:1696–1708. <http://dx.doi.org/10.1038/sj.onc.1209327>.
- Pietras A, Katz AM, Ekström EJ, Wee B, Halliday JJ, Pitter KL, Werbeck JL, Amankulor NM, Huse JT, Holland EC. 2014. Osteopontin-CD44 signaling in the glioma perivascular niche enhances cancer stem cell phenotypes and promotes aggressive tumor growth. *Cell Stem Cell* 14:357–369. <http://dx.doi.org/10.1016/j.stem.2014.01.005>.
- Todaro M, Gaggiani M, Catalano V, Benfante A, Iovino F, Biffoni M, Apuzzo T, Sperduti I, Volpe S, Cocorullo G, Gulotta G, Dieli F, De Maria R, Stassi G. 2014. CD44v6 is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. *Cell Stem Cell* 14:342–356. <http://dx.doi.org/10.1016/j.stem.2014.01.009>.
- Godar S, Ince TA, Bell GW, Feldser D, Donaher JL, Bergh J, Liu A, Miu K, Watnick RS, Reinhardt F, McAllister SS, Jacks T, Weinberg RA. 2008. Growth-inhibitory and tumor-suppressive functions of p53 depend on its repression of CD44 expression. *Cell* 134:62–73. <http://dx.doi.org/10.1016/j.cell.2008.06.006>.
- Matzke A, Herrlich P, Ponta H, Orian-Rousseau V. 2005. A five-amino-acid peptide blocks Met- and Ron-dependent cell migration. *Cancer Res* 65:6105–6110. <http://dx.doi.org/10.1158/0008-5472.CAN-05-0207>.
- Günther U, Hofmann M, Rudy W, Reber S, Zöller M, Haussmann I, Matzku S, Wenzel A, Ponta H, Herrlich P. 1991. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 65:13–24. [http://dx.doi.org/10.1016/0092-8674\(91\)90403-L](http://dx.doi.org/10.1016/0092-8674(91)90403-L).
- Yu Q, Toole BP, Stamenkovic I. 1997. Induction of apoptosis of metastatic mammary carcinoma cells in vivo by disruption of tumor cell surface CD44 function. *J Exp Med* 186:1985–1996. <http://dx.doi.org/10.1084/jem.186.12.1985>.
- Zeilstra J, Joosten SPJ, van Andel H, Tolg C, Berns A, Snoek M, van de Wetering M, Spaargaren M, Clevers H, Pals ST. 2014. Stem cell CD44v isoforms promote intestinal cancer formation in Apc(min) mice downstream of Wnt signaling. *Oncogene* 33:665–670. <http://dx.doi.org/10.1038/ncr.2012.611>.
- Sherman L, Wainwright D, Ponta H, Herrlich P. 1998. A splice variant of CD44 expressed in the apical ectodermal ridge presents fibroblast growth factors to limb mesenchyme and is required for limb outgrowth. *Genes Dev* 12:1058–1071. <http://dx.doi.org/10.1101/gad.12.7.1058>.
- Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H. 2002. CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev* 16:3074–3086. <http://dx.doi.org/10.1101/gad.242602>.
- Lautrette A, Li S, Alili R, Sunnarborg SW, Burtin M, Lee DC, Friedlander G, Terzi F. 2005. Angiotensin II and EGF receptor cross-talk in chronic kidney diseases: a new therapeutic approach. *Nat Med* 11:867–874. <http://dx.doi.org/10.1038/nm1275>.
- Sternlicht MD, Sunnarborg SW. 2008. The ADAM17-amphiregulin-EGFR axis in mammary development and cancer. *J Mammary Gland Biol Neoplasia* 13:181–194. <http://dx.doi.org/10.1007/s10911-008-9084-6>.
- Perugorria MJ, Latasa MU, Nicou A, Cartagena-Lirola H, Castillo J, Goñi S, Vespasiani-Gentilucci U, Zagami MG, Lotersztajn S, Prieto J, Berasain C, Avila MA. 2008. The epidermal growth factor receptor ligand amphiregulin participates in the development of mouse liver fibrosis. *Hepatology* 48:1251–1261. <http://dx.doi.org/10.1002/hep.22437>.
- Iwamoto R, Yamazaki S, Asakura M, Takashima S, Hasuwa H, Miyado K, Adachi S, Kitakaze M, Hashimoto K, Raab G, Nanba D, Higashiyama S, Hori M, Klagsbrun M, Mekada E. 2003. Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. *Proc Natl Acad Sci U S A* 100:3221–3226. <http://dx.doi.org/10.1073/pnas.0537588100>.
- Kveiborg M, Instrell R, Rowlands C, Howell M, Parker PJ. 2011. PKCα and PKCδ regulate ADAM17-mediated ectodomain shedding of heparin binding-EGF through separate pathways. *PLoS One* 6:e17168. <http://dx.doi.org/10.1371/journal.pone.0017168>.
- Dang M, Dubbin K, D'Aiello A, Hartmann M, Lodish H, Herrlich A. 2011. Epidermal growth factor (EGF) ligand release by substrate-specific a disintegrin and metalloproteases (ADAMs) involves different protein kinase C (PKC) isoenzymes depending on the stimulus. *J Biol Chem* 286: 17704–17713. <http://dx.doi.org/10.1074/jbc.M110.187823>.
- Le Gall SM, Maretzky T, Issuree PDA, Niu X-D, Reiss K, Saftig P, Khokha R, Lundell D, Blobel CP. 2010. ADAM17 is regulated by a rapid and reversible mechanism that controls access to its catalytic site. *J Cell Sci* 123:3913–3922. <http://dx.doi.org/10.1242/jcs.069997>.
- Düsterhöft S, Höbel K, Oldefest M, Lokau J, Waetzig GH, Chalaris A, Garbers C, Scheller J, Rose-John S, Lorenzen I, Grötzinger J. 2014. A disintegrin and metalloprotease 17 dynamic interaction sequence, the

- sweet tooth for the human interleukin 6 receptor. *J Biol Chem* 289:16336–16348. <http://dx.doi.org/10.1074/jbc.M114.557322>.
32. Düsterhöft S, Jung S, Hung C-W, Tholey A, Sonnichsen FD, Grötzinger J, Lorenzen I. 2013. Membrane-proximal domain of a disintegrin and metalloprotease-17 represents the putative molecular switch of its shedding activity operated by protein-disulfide isomerase. *J Am Chem Soc* 135:5776–5781. <http://dx.doi.org/10.1021/ja400340u>.
 33. Willems SH, Tape CJ, Stanley PL, Taylor NA, Mills IG, Neal DE, McCafferty J, Murphy G. 2010. Thiol isomerases negatively regulate the cellular shedding activity of ADAM17. *Biochem J* 428:439–450. <http://dx.doi.org/10.1042/BJ20100179>.
 34. Xu P, Liu J, Sakaki-Yumoto M, Derynck R. 2012. TACE activation by MAPK-mediated regulation of cell surface dimerization and TIMP3 association. *Sci Signal* 5:ra34. <http://dx.doi.org/10.1126/scisignal.2002689>.
 35. Xu P, Derynck R. 2010. Direct activation of TACE-mediated ectodomain shedding by p38 MAP kinase regulates EGF receptor-dependent cell proliferation. *Mol Cell* 37:551–566. <http://dx.doi.org/10.1016/j.molcel.2010.01.034>.
 36. Herrlich A, Klinman E, Fu J, Sadegh C, Lodish H. 2008. Ectodomain cleavage of the EGF ligands HB-EGF, neuregulin-1-beta, and TGF-alpha is specifically triggered by different stimuli and involves different PKC isoenzymes. *FASEB J* 22:4281–4295. <http://dx.doi.org/10.1096/fj.08-113852>.
 37. Sahin U, Weskamp G, Kelly K, Zhou HM, Higashiyama S, Peschon J, Hartmann D, Saftig P, Blobel CP. 2004. Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J Cell Biol* 164:769–779. <http://dx.doi.org/10.1083/jcb.200307137>.
 38. Parra LM, Zou Y. 2010. Sonic hedgehog induces response of commissural axons to semaphorin repulsion during midline crossing. *Nat Neurosci* 13:29–35. <http://dx.doi.org/10.1038/nn.2457>.
 39. Lillesaar C, Fried K. 2004. Neurites from trigeminal ganglion explants grown in vitro are repelled or attracted by tooth-related tissues depending on developmental stage. *Neuroscience* 125:149–161. <http://dx.doi.org/10.1016/j.neuroscience.2004.01.008>.
 40. Torres-Espín A, Santos D, González-Pérez F, Del Valle J, Navarro X. 2014. Neurite-J: an Image-J plug-in for axonal growth analysis in organotypic cultures. *J Neurosci methods* 236:26–39. <http://dx.doi.org/10.1016/j.jneumeth.2014.08.005>.
 41. Hartmann M, Parra LM, Ruschel A, Lindner C, Morrison H, Herrlich A, Herrlich P. 2015. Inside-out regulation of ectodomain cleavage of cluster-of-differentiation-44 (CD44) and of neuregulin-1 requires substrate dimerization. *J Biol Chem* 290:17041–17054. <http://dx.doi.org/10.1074/jbc.M114.610204>.
 42. Bollen M, Peti W, Ragusa MJ, Beullens M. 2010. The extended PP1 toolkit: designed to create specificity. *Trends Biochem Sci* 35:450–458. <http://dx.doi.org/10.1016/j.tibs.2010.03.002>.
 43. Mori T, Kitano K, Terawaki S-i, Maesaki R, Fukami Y, Hakoshima T. 2008. Structural basis for CD44 recognition by ERM proteins. *J Biol Chem* 283:29602–29612. <http://dx.doi.org/10.1074/jbc.M803606200>.
 44. Fricker FR, Antunes-Martins A, Galino J, Paramsothy R, La Russa F, Perkins J, Goldberger R, Brelstaff J, Zhu N, McMahon SB, Orenge C, Garratt AN, Birchmeier C, Bennett DLH. 2013. Axonal neuregulin 1 is a rate limiting but not essential factor for nerve remyelination. *Brain* 136:2279–2297. <http://dx.doi.org/10.1093/brain/awt148>.
 45. Meyer D, Yamaai T, Garratt A, Riethmacher-Sonnenberg E, Kane D, Theill LE, Birchmeier C. 1997. Isoform-specific expression and function of neuregulin. *Development* 124:3575–3586.
 46. Birchmeier C, Nave K-A. 2008. Neuregulin-1, a key axonal signal that drives Schwann cell growth and differentiation. *Glia* 56:1491–1497. <http://dx.doi.org/10.1002/glia.20753>.
 47. Brinkmann BG, Agarwal A, Sereda MW, Garratt AN, Müller T, Wende H, Stassart RM, Nawaz S, Humml C, Velanac V, Radyushkin K, Goebbels S, Fischer TM, Franklin RJ, Lai C, Ehrenreich H, Birchmeier C, Schwab MH, Nave KA. 2008. Neuregulin-1/Erbb signaling serves distinct functions in myelination of the peripheral and central nervous system. *Neuron* 59:581–595. <http://dx.doi.org/10.1016/j.neuron.2008.06.028>.
 48. Liu X, Hwang H, Cao L, Buckland M, Cunningham A, Chen J, Chien KR, Graham RM, Zhou M. 1998. Domain-specific gene disruption reveals critical regulation of neuregulin signaling by its cytoplasmic tail. *Proc Natl Acad Sci U S A* 95:13024–13029. <http://dx.doi.org/10.1073/pnas.95.22.13024>.
 49. Liu X, Hwang H, Cao L, Wen D, Liu M, Graham RM, Zhou M. 1998. Release of the neuregulin functional polypeptide requires its cytoplasmic tail. *J Biol Chem* 273:34335–34340. <http://dx.doi.org/10.1074/jbc.273.51.34335>.
 50. Esper RM, Loeb JA. 2009. Neurotrophins induce neuregulin release through protein kinase C δ activation. *J Biol Chem* 284:26251–26260. <http://dx.doi.org/10.1074/jbc.M109.002915>.
 51. Kahn J, Walcheck B, Migaki GI, Jutila MA, Kishimoto TK. 1998. Calmodulin regulates L-selectin adhesion molecule expression and function through a protease-dependent mechanism. *Cell* 92:809–818. [http://dx.doi.org/10.1016/S0092-8674\(00\)81408-7](http://dx.doi.org/10.1016/S0092-8674(00)81408-7).
 52. Matala E, Alexander SR, Kishimoto TK, Walcheck B. 2001. The cytoplasmic domain of L-selectin participates in regulating L-selectin endoproteolysis. *J Immunol* 167:1617–1623. <http://dx.doi.org/10.4049/jimmunol.167.3.1617>.
 53. Killock DJ, Ivetić A. 2010. The cytoplasmic domains of TNF α -converting enzyme (TACE/ADAM17) and L-selectin are regulated differently by p38 MAPK and PKC to promote ectodomain shedding. *Biochem J* 428:293–304. <http://dx.doi.org/10.1042/BJ20091611>.
 54. Darmellah A, Rayah A, Auger R, Cuif M-H, Prigent M, Arpin M, Alcover A, Delarasse C, Kanellopoulos JM. 2012. Ezrin/radixin/moesin are required for the purinergic P2X7 receptor (P2X7R)-dependent processing of the amyloid precursor protein. *J Biol Chem* 287:34583–34595. <http://dx.doi.org/10.1074/jbc.M112.400010>.
 55. Kohlstedt K, Shoghi F, Müller-Esterl W, Busse R, Fleming I. 2002. CK2 phosphorylates the angiotensin-converting enzyme and regulates its retention in the endothelial cell plasma membrane. *Circ Res* 91:749–756. <http://dx.doi.org/10.1161/01.RES.0000038114.17939.C8>.
 56. Müllberg J, Oberthür W, Lottspeich F, Mehl E, Dittrich E, Graeve L, Heinrich PC, Rose-John S. 1994. The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site. *J Immunol* 152:4958–4968.
 57. Crowe PD, VanArsdale TL, Goodwin RG, Ware CF. 1993. Specific induction of 80-kDa tumor necrosis factor receptor shedding in T lymphocytes involves the cytoplasmic domain and phosphorylation. *J Immunol* 151:6882–6890.
 58. Wang X, Mizushima H, Adachi S, Ohishi M, Iwamoto R, Mekada E. 2006. Cytoplasmic domain phosphorylation of heparin-binding EGF-like growth factor. *Cell Struct Funct* 31:15–27. <http://dx.doi.org/10.1247/csf.31.15>.
 59. Maretzky T, Evers A, Le Gall S, Alabi RO, Speck N, Reiss K, Blobel CP. 2015. The cytoplasmic domain of a disintegrin and metalloproteinase 10 (ADAM10) regulates its constitutive activity but is dispensable for stimulated ADAM10-dependent shedding. *J Biol Chem* 290:7416–7425. <http://dx.doi.org/10.1074/jbc.M114.603753>.
 60. Schwarz J, Broder C, Helmstetter A, Schmidt S, Yan I, Müller M, Schmidt-Arras D, Becker-Pauly C, Koch-Nolte F, Mitrücker H-W, Rabe B, Rose-John S, Chalaris A. 2013. Short-term TNF α shedding is independent of cytoplasmic phosphorylation or furin cleavage of ADAM17. *Biochim Biophys Acta* 1833:3355–3367. <http://dx.doi.org/10.1016/j.bbamer.2013.10.005>.
 61. Reddy P, Slack JL, Davis R, Cerretti DP, Kozlosky CJ, Blanton RA, Shows D, Peschon JJ, Black RA. 2000. Functional analysis of the domain structure of tumor necrosis factor- α converting enzyme. *J Biol Chem* 275:14608–14614. <http://dx.doi.org/10.1074/jbc.275.19.14608>.
 62. Maretzky T, McIlwain DR, Issuree PDA, Li X, Malapeira J, Amin S, Lang PA, Mak TW, Blobel CP. 2013. iRhom2 controls the substrate selectivity of stimulated ADAM17-dependent ectodomain shedding. *Proc Natl Acad Sci U S A* 110:11433–11438. <http://dx.doi.org/10.1073/pnas.1302553110>.
 63. Tsukamoto H, Tanida S, Ozeki K, Ebi M, Mizoshita T, Shimura T, Mori Y, Kataoka H, Kamiya T, Fukuda S, Higashiyama S, Joh T. 2013. Annexin A2 regulates a disintegrin and metalloproteinase 17-mediated ectodomain shedding of pro-tumor necrosis factor- α in monocytes and colon epithelial cells. *Inflamm Bowel Dis* 19:1365–1373. <http://dx.doi.org/10.1097/MIB.0b013e318281f43a>.
 64. Nakayama H, Fukuda S, Inoue H, Nishida-Fukuda H, Shirakata Y, Hashimoto K, Higashiyama S. 2012. Cell surface annexins regulate ADAM-mediated ectodomain shedding of proamphiregulin. *Mol Biol Cell* 23:1964–1975. <http://dx.doi.org/10.1091/mbc.E11-08-0683>.
 65. Legg JW, Lewis CA, Parsons M, Ng T, Isacke CM. 2002. A novel PKC-regulated mechanism controls CD44 ezrin association and directional cell motility. *Nat Cell Biol* 4:399–407. <http://dx.doi.org/10.1038/ncb797>.
 66. Fridman JS, Caulder E, Hansbury M, Liu X, Yang G, Wang Q, Lo Y,

- Zhou BB, Pan M, Thomas SM, Grandis JR, Zhuo J, Yao W, Newton RC, Friedman SM, Scherle PA, Vaddi K. 2007. Selective inhibition of ADAM metalloproteases as a novel approach for modulating ErbB pathways in cancer. *Clin Cancer Res* 13:1892–1902. <http://dx.doi.org/10.1158/1078-0432.CCR-06-2116>.
67. Friedman S, Levy R, Garrett W, Doval D, Bondarde S, Sahoo T, Lokanatha D, Julka P, Shenoy K, Nagarkar R, Bhattacharyya G, Kumar K, Nag S, Mohan P, Narang N, Raghunadharao D, Walia M, Yao W, Li J, Emm T, Yeleswaram S, Scherle P, Newton R. 2009. Clinical benefit of INCB7839, a potent and selective inhibitor of ADAM10 and ADAM17, in combination with trastuzumab in metastatic HER2-positive breast cancer patients. *Cancer Res* 69(Suppl):5056.