Tumor Suppressor NF2 Blocks Cellular Migration by Inhibiting Ectodomain Cleavage of CD44

Monika Hartmann1, Liseth M. Parra1,2, Anne Ruschel1, Sandra Böhme1, Yong Li1, Helen Morrison1, Andreas Herrlich2, and Peter Herrlich1

Abstract

Ectodomain cleavage (shedding) of transmembrane proteins by metalloproteases (MMP) generates numerous essential signaling molecules, but its regulation is not totally understood. CD44, a cleaved transmembrane glycoprotein, exerts both anti-proliferative or tumor-promoting functions, but whether proteolysis is required for this is not certain. CD44-mediated contact inhibition and cellular proliferation are regulated by counteracting CD44 C-terminal interacting proteins, the tumor suppressor protein merlin (NF2) and ERM proteins (ezrin, radixin, moesin). We show here that activation or overexpression of constitutively active merlin or downregulation of ERM proteins in inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced as well as serum, hepatocyte growth factor (HGF), or platelet-derived growth factor (PDGF) CD44 cleavage by the metalloprotease ADAM10, whereas overexpressed ERM proteins promoted cleavage. Merlin- and ERM-modulated Ras or Rac activity was not required for this function. However, latrunculin (an actin-disrupting toxin) or an ezrin mutant which is unable to link CD44 to actin, inhibited CD44 cleavage, identifying a cytoskeletal C-terminal link as essential for induced CD44 cleavage. Cellular migration, an important tumor property, depended on CD44 and its cleavage and was inhibited by merlin. These data reveal a novel function of merlin and suggest that CD44 cleavage products play a tumor-promoting role. Neuregulin, an EGF ligand released by ADAM17 from its pro-form NRG1, is predominantly involved in regulating cellular differentiation. In contrast to CD44, release of neuregulin from its pro-form was not regulated by merlin or ERM proteins. Disruption of the actin cytoskeleton however, also inhibited NRG1 cleavage. This current study presents one of the first examples of substrate-selective cleavage regulation.

Implications: Investigating transmembrane protein cleavage and their regulatory pathways have provided new molecular insight into their important role in cancer formation and possible treatment. Mol Cancer Res; 13(5); 879–90. ©2015 AACR.

Introduction

The ubiquitously expressed surface glycoprotein CD44 is involved in a number of cellular functions not all of which are understood in molecular terms. Its role in cell-cycle control has obtained most attention and it seems mechanically best understood. Depending on extracellular ligands, intracellular partner proteins, and/or the inclusion of alternatively spliced exon sequences, CD44 can act as a tumor suppressor and mediate contact inhibition or can act alternatively as a tumor promoter and metastasis inducer. Binding of high-molecular-weight hyaluronan causes cell-cycle arrest and tumor suppression (1–3). To achieve cell-cycle arrest, the tumor suppressor protein merlin (neurofibromatosis type 2; NF2) is recruited to the cytoplasmic tail of CD44, a location from which it inhibits Ras- and Rac-dependent signaling (1, 4). On the other hand, CD44 can counteract the tumor suppressor p53. In order for p53 to act as a tumor suppressor, CD44 expression needs to be downregulated (5). In addition, CD44 acts as coreceptor for receptor tyrosine kinases, the most prominent example being c-Met which depends on the presence of a CD44 splice variant comprising exon v6 (6). This second function of CD44 promotes tumor growth and metastasis formation (7–9).

The tumor suppressor protein merlin (NF2), like CD44, is ubiquitously expressed in mammals. Mice carrying one mutated nf2 allele are at risk of developing several types of tumors (10). Merlin is kept inactive in proliferating cells but is activated by phosphorylation of 2 serines upon cell–cell contact and/or hyaluronan binding (ref. 2 and unpublished data). Dephosphorylation is regulated by a signal transduction pathway emerging from cell–cell and/or hyaluronan–cell contact and involves all ERM proteins in addition to merlin. Interestingly, while merlin is activated by this process, the ERM proteins are inactivated (11–13). Several types of analyses have led to proposals on how merlin could act as a tumor suppressor, for example, by inhibiting mitogenic signaling, activating the Hippo pathway, and/or promoting the establishment of adherens junctions (reviewed by ref. 14).

Several years ago, it was discovered that CD44—like numerous other transmembrane proteins—is subject to ectodomain cleavage by metalloprotease activity (15, 16), now identified as ADAM10 (a...
disintegrin and metalloprotease 10; refs. 17–19). This is followed by γ-secretase–dependent release of the cytoplasmic tail, which promotes the expression of proliferation-promoting genes in the nucleus. Given the tumor-suppressive role of hyaluronan-bound CD44, ectodomain cleavage would abolish this function. We therefore investigated how ectodomain cleavage of CD44 might be regulated.

We report here that it is the tumor suppressor protein merlin itself that prevents CD44 cleavage, supporting the notion that proteolytic processing of CD44 promotes tumor growth and the hypothesis that naturally occurring Nf2 mutants that are prone to malignancies may fail to inhibit CD44 ectodomain cleavage and thereby its tumor-promoting role. This cleavage regulation is specific to CD44, as we show that NRG1, the pro-form of the EGF ligand neuregulin, an ADAM17 substrate and major regulator of cellular differentiation, is cleaved upon stimulation but is not regulated by merlin or ERM.

**Materials and Methods**

**Reagents**

DNA oligonucleotides (Metabion GmbH); 12-O-tetradecanoyl-phorbol-13-acetate (TPA); DAPT, latrunculin B, and batimastat (Sigma); PD98059 (Cell Signaling Technology); angiotensin II (ARIAD Pharmaceuticals, Inc.), 4-OH tamoxifen, EGF, FGF (Sigma); hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF; R&D Systems); and CK-548 (Tocris).

**Antibodies**

Anti-FLAG (M2 and SIG1-25; Sigma) and phosphospecific antibodies against ezrin (Thr567)/radixin (Thr564)/moesin (Thr568) and p44/42 MAPK (Thr202/Tyr204; Cell Signaling Technology); ADAM10 (735–303)-tubulin (Abcam). Antibodies directed against human CD44: for the standard isoform of rat CD44 was provided by Christoph Kaether (http://www.imb-jena.de/”; Fritz Lipmann Institute - Leibniz Institute for Age Research, Jena, Germany)). All secondary antibodies were from Dako. Human moesin NM_002444 AGAUCGAGGAACAGACUAAGA Human radixin NM_002903 AAGCAGUUGGAAAGGGCACAA Human ezrin NM_003379 AACCCCAAAGAUUGGCUUUCC Luciferase (control) CGUACGCGGAAUACUUCGATT Human ADAM10 NM_001110 GCUAAUGGCUGGAUUUAUU Other siRNA oligonucleotides were from Applied Biosystems/Ambion. The sequences of the siRNA oligonucleotides are listed in Table 2. For downregulation of ERM proteins, a mixture of oligonucleotides targeting ezrin, radixin, and moesin was used.

**Inhibition of cleavage conditions**

Metalloprotease activity was blocked with 5 μmol/L batimastat (BB94; Calbiochem) 15 minutes before TPA stimulation. γ-Secretase activity was blocked by 5 μmol/L DAPT (Sigma) or by 10 μmol/L compound E (Enzo).

**Cell migration assays—scratch wound assay**

We isolated mouse embryonic fibroblasts from mice with cd44lox/lox [cd44fl/fl], GT(Rosa)26-CRE (B6/129)] and immortalized these by downregulation of p19ARF. Cd44 gene deletion was achieved by treatment with tamoxifen. Scratch wound assays were performed in triplicates in 6-well plates at high cell density (1.5 × 10⁵ cells per well). Twenty-four hours after seeding, cells were serum-starved for another 24 hours. Scratches were introduced with a 200-μl pipette tip and cultures were resupplied with serum-containing medium. Where indicated, cleavage was inhibited by adding 5 μmol/L batimastat. Scratches were imaged at 10, 24, and 36 hours after scratching. Wound areas were quantified using Photoshop and ImageJ software.

**Statistical analysis**

Intensity of immunoblot bands was quantified using ImageJ and Image Lab (BioRad) software. All values on histograms are reported as mean ± SD, P < 0.05 (Student t test) was considered significant.

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**Table 1. Primers used for mutagenesis**

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See Supplementary Data for Cell lines, transfections, TCA-DOC precipitations, coimmunoprecipitation, generation of cell lysates and analysis

Results

Increased cell density inhibits ectodomain cleavage of CD44

As has been reported previously, CD44 is subject to the metalloprotease ADAM10-dependent ectodomain cleavage and subsequent release of the cytoplasmic CD44 C-terminus by γ-secretase (17–19). Aiming at understanding the regulation of CD44 ectodomain cleavage, we introduced expression constructs encoding doubly tagged (N- and C-terminal tags) CD44 proteins into NIH3T3, CD44 Flag−/−MEFs, RPM-MC, or MDA-MB-231 cells and examined their proteolytic processing. The N-terminus of CD44 carried a FLAG tag, the C-terminus, a c-myc motif. RPM-MC human melanoma cells, as well as CD44 Flag−/−MEFs, do not express endogenous CD44, which simplified detection of the transfected molecule, permitted to introduce CD44 mutants, and allowed to analyze signaling pathways independently of endogenous CD44. In NIH3T3 and MDA-MD-231 cells, we also investigated the cleavage of endogenous CD44. NRG1 was similarly tagged by N-terminal FLAG and C-terminal myc tag. CD44 and NRG1 cleavage was induced by TPA, a phorbol ester that mimics diacylglycerol and activates most protein kinase C (PKC) isozymes. Cleavage could also be induced by serum factors, by HGF and cysglycerol and activates most protein kinase C (PKC) isozymes. Cleavage could also be induced by serum factors, by HGF and PDGF, and, if cells carried the appropriate G-protein–coupled receptor, by angiotensin II. In most experiments, γ-secretase activity was blocked using the γ-secretase inhibitor, DAPT, to quantitate only the products of the first processing event, ADAM-dependent ectodomain cleavage. Omission of DAPT did not significantly alter our principal results on regulation, but caused further processing of the C-terminal ADAM-dependent cleavage product (Supplementary Fig. S1). It is important to note that in our analysis of CD44 and NRG1 cleavage, we ensured that we focused exclusively on processing of the substrates after their proper insertion into the plasma membrane. To ascertain this, we carried out experiments very shortly after cell surface biotinylation, showing that biotinylated ectodomains, solCD44E and neurategulin, are indeed released into the supernatant (Supplementary, showing that biotinylated ectodomains, solCD44E and neurategulin, are indeed released into the supernatant (Supplementary, showing that biotinylated ectodomains, solCD44E and neurategulin, are indeed released into the supernatant (Supplementary, showing that biotinylated ectodomains, solCD44E and neurategulin, are indeed released into the supernatant (Supplementary, showing that biotinylated ectodomains, solCD44E and neurategulin, are indeed released into the supernatant (Supplementary) and their fluorescence was specifically blocked by batimastat (Fig. 3A, lane 1 vs. 3).

Because CD44 regulates contact inhibition of cells, we wondered whether its cleavage regulation was dependent on cell density. While spontaneous cleavage was reduced only slightly, TPA-induced cleavage was markedly diminished by increasing cell density from 5 × 10^4 to 9 × 10^5 cells per well of a 6-well plate (for quantitation, see the column diagram of 3 independent experiments; Fig. 1C). From previous reports, it has been known that high cell density causes dephosphorylation of both merlin and its counterplayers, the ezrin–moesin–radixin (ERM) proteins, by the same protein phosphatase-1 isozyme (2). Dephosphorylation of ERM proteins deactivates them whereas it activates merlin. In turn, phosphorylation of both ERM proteins and merlin depends on protein kinase activity during the exponential growth of cells (21–23). While little phospho-ERM could be detected in the absence of a stimulus (left 3 lanes in Fig. 1C), ERM proteins were strongly phosphorylated upon TPA treatment of cells (compare lanes 1 and 4, Fig. 1C). As expected, phosphorylation of ERM proteins declined with increasing cell density, coinciding with decreased CD44 cleavage (lanes 5 and 6, Fig. 1C). Merlin dephosphorylation follows exactly that of the ERM proteins (data not shown and Supplementary Fig. S3A).

The tumor suppressor protein merlin inhibits ectodomain cleavage of CD44

To investigate whether dephosphorylation of ERM proteins and reduced CD44 ectodomain cleavage with increasing cell density was not simply coincidental, we tested the effect of over-expression of merlin or of ERM mutants (see below) on CD44-induced cleavage in RPM-MC cells. To this end, we first examined the effect of a constitutively active merlin mutant (NF2-S518A), which does not require dephosphorylation. These experiments were done under low cell density conditions at which endogenous merlin is phosphorylated and inactive, and activated ERM proteins drive proliferation. TPA induced solCD44E release in the absence of transfected merlin (Fig. 2A; compare lanes 1 and 4, WB: FLAG). However, expression of the singly mutated active merlin (NF2-S518A) was sufficient to inhibit solCD44E release in RPM-MC cells (Fig. 2A; compare lanes 2 and 5, WB: FLAG; also see column diagram of quantification of 3 independent experiments), whereas the phospho-mimicking mutant S518D had no effect (see also similar data obtained in NIH3T3 cells, Supplementary Fig. S4). Cleavage of the ADAM17 substrate neurategulin (NRG1) in the same cells was not inhibited (Fig. 2B). Quantitations have been compiled as column diagram in Fig. 2A/B. We can therefore conclude that the tumor suppressor merlin specifically inhibits CD44 cleavage by ADAM10 and that merlin does not interfere with a common signaling pathway addressing ADAM cleavage in general.

We had previously shown that contact inhibition requires the binding of dephosphorylated active merlin to the C-terminus of CD44 via a membrane-proximal basic amino acid sequence, known as the KR motif (Supplementary Fig. S3B; initially described as an ezrin-binding site; ref. 1). We therefore followed the idea that cleavage regulation of CD44 might also require merlin binding to the C-terminus. To investigate this, we compared cleavage of wild-type CD44 and the CD44 mutant with a complete deletion of the intracellular domain, ICD (CD44ΔICD). First, we made sure, by confocal microscopy and immunostaining, that the deletion mutant was properly inserted into the plasma membrane (data not shown). Second, we confirmed that it was still processed by ADAM10 (as is CD44 art). Release of solCD44E from CD44ΔICD was blocked by siRNA-dependent downregulation of ADAM10 (Fig. 3A, compare lanes 1 and 2) or by addition of the ADAM inhibitor batimastat (Fig. 3A, lane 1 vs. 3).
We then compared the action of merlin mutants on cleavage of full-length CD44 (WtCD44; first six lanes, Fig. 3B) and of CD44 with deletion of the ICD (CD44\textsubscript{D\textsubscript{ICD}}; lanes 7–12, Fig. 3B; see also the top column diagram and the loading scheme in Supplementary Fig. S7) as well as on cleavage of the noncleaved mutant CD44-KR-Mt (bottom column diagram, Fig. 3B). Indeed, absence of the entire CD44 intracellular domain as well as mutation of the KR motif prevented the inhibitory effect of merlin on cleavage.

The topmost first panel in Fig. 3B, shows the level of endogenous (inactive) merlin and of the transfected merlin mutants. The third panel shows detection of the released solCD44E and the fourth panel the full-length molecules (both detected with anti-FLAG antibodies). As already shown in Fig. 2, constitutively active NF2-S518A, but not NF2-S518D, inhibited full-length CD44 cleavage (lanes 5 and 6, Fig. 3B). Interestingly, total absence of the cytoplasmic tail of CD44 caused significant spontaneous ectodomain cleavage (lane 7, Fig. 3B), suggesting that the ICD suppressed spontaneous cleavage and was required for TPA-induced regulation. However, TPA was still able to increase cleavage to some extent (compare lanes 7 and 10, Fig. 3B; also see Discussion). Both spontaneous and induced solCD44E release from the CD44\textsubscript{D\textsubscript{ICD}} mutant was resistant to inhibition by constitutively active merlin NF2-S518A (compare lanes 8 and 11, Fig. 3B). Note that NF2-S518D had little to no effect on this release (lanes 9 and 12, Fig. 3B). A quantitation of 3 independent experiments with CD44\textsubscript{D\textsubscript{ICD}} is shown in the top column diagram. Strong cleavage coincided with the appearance of several solCD44E bands (Fig. 3A and B). We assume that this might be due to the presence of several ADAM10 cleavage sites on CD44. The CD44KR-Mt mutant was barely inducible by TPA and not inhibited by merlin (see bottom column diagram in Fig. 3B), as one would expect because the mutation destroys the merlin-binding site. For comparison, another ADAM substrate, the amyloid precursor protein, APP, is shown in the second panel of Fig. 3B. TPA induced cleavage of both CD44 and of APP (compare lanes 1 and 4, Fig. 3B); however, cleavage of the ADAM17 substrate APP was not affected by merlin.

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Figure 1.
Cell density–dependent regulation of CD44 ectodomain cleavage. A, TPA induced ADAM-dependent ectodomain cleavage of CD44. The CD44-negative cell line RPM-MC was transfected with a doubly tagged expression construct of standard isoform of wild-type CD44 (CD44\textsubscript{s}). V, vector control. All samples were treated with γ-secretase inhibitor DAPT (5 μmol/L). Batimastat (5 μmol/L) was added as indicated. The cells were kept in logarithmic growth condition. Full-length CD44 (CD44\textsubscript{fl}) was detected by an antibody recognizing the C-terminal myc tag. The cleaved ectodomain (solCD44E) was detected in culture supernatants after TCA-DOC (see Supplementary Materials and Methods) precipitation by an antibody against the N-terminal FLAG tag. Treatment of the cells with 100 ng/mL phorbol ester (TPA) induced detectable cleavage within 15 minutes and led to accumulation of solCD44E in 3 to 4 hours. Here, TPA treatment was for 3 hours. Actin served as loading control. The cleavage is inhibited by the metalloprotease inhibitor batimastat. B, TPA induced ADAM-dependent ectodomain cleavage of CD44 in the absence of γ-secretase inhibition (no DAPT). C, high cell density inhibits ectodomain cleavage of CD44. RPM-MC cells transfected with tagged CD44 as in A were seeded into 6-well plates at different densities, as indicated. Cells were treated with 100 ng/mL TPA for 4 hours. The amount of cell lysates loaded on the gel was normalized to actin levels. CD44 cleavage was detected by the release of CD44\textsubscript{AE} (membrane-bound cleavage product lacking the ectodomain) and by c-Myc immunoblot in cell lysates. TPA-induced generation of the membrane-bound cleavage product (CD44\textsubscript{AE}) was diminished with increasing cell density. At the same time, merlin (not shown) and the ERM proteins were dephosphorylated (middle). The 62-kDa band (†) likely represents endogenous c-Myc; the 40-kDa band (‡) is unspecific. Representative blots are shown. The intensity of immunoblot bands was quantified with ImageJ. Histograms show mean values of relative level of cleavage ± SD from 3 independent experiments (**, P = 0.000924 for 5 × 10\textsuperscript{5} vs. 7 × 10\textsuperscript{5} and P = 0.000211 for 5 × 10\textsuperscript{5} vs. 9 × 10\textsuperscript{5}). Levels of phospho-ERM proteins relative to that at the density of 5 × 10\textsuperscript{5} are indicated within the immunoblot.
(compare lanes 5, 6 and 11, 12, Fig. 3B). Also, cleavage of the ADAM10 substrate c-Met was merlin-resistant (data not shown). Consistent with this neither APP nor c-Met does, to our knowledge, carry a KR motif in its C-terminus.

Our results using the CD44 ICD deletion and the KR-Mt mutant suggest that merlin interaction with the ICD of CD44 is necessary for its cleavage regulating function. Importantly, CD44 was coprecipitated with merlin only if its ICD was intact (Fig. 3C). Mutation of the KR motif abolished merlin interaction (Supplementary Fig. S3B), identical to the ICD deletion (Fig. 3C). Similarly, the absence of merlin regulation on neuregulin release (Fig. 2B) and the resistance of APP, c-Met, and NRG1 cleavage to regulation by merlin further strengthens the idea of substrate-specific regulation of ectodomain cleavage and confirms our assertion that merlin exerts a direct specific effect on CD44 and its cleavage, rather than interfering with a cleavage regulatory signaling pathway common to these 3 substrates.

The action of ERM proteins and the mechanism of merlin-dependent inhibition of CD44 ectodomain cleavage

CD44 C-terminally bound merlin mediates contact inhibition predominantly by blocking Ras and Rac activity. This is counteracted by ERM proteins, which promote Ras and Rac activation. A well-studied mechanism has shown that Ras is activated by ERM proteins interacting with both Ras and the guanine nucleotide exchange factor and activator of Ras, son of sevenless (SOS; ref. 24). Because active merlin inhibits CD44 ectodomain cleavage, we were wondering whether its Ras inhibitory action was required for this effect and in turn whether cleavage required ERM protein-dependent Ras activation.

Upon downregulation of all 3 ERM proteins using siRNA, TPA-induced cleavage was indeed significantly reduced [detected by the reduced release of solCD44E (N-terminal FLAG tag) and of the membrane-bound cleavage product CD44D; compare lanes 2 and 5]. The phospho-merlin mimicking inactive mutant NFS518D did not affect the cleavage induction (lanes 3 and 6). Because of the similar migration of merlin and CD44, these 2 proteins were detected on separate gels, and respectively 2 subsequent loading controls are shown. B, corresponding experiment as in (A) shows the result for another ADAM substrate, the neuregulin precursor NRG1. Merlin-dependent inhibition was specific for CD44, as NRG1 was not affected. The histograms in A/B show mean values of relative level of cleavage ±SD from 3 independent experiments (**, P = 0.000537).
in Fig. 4C; see also the experimental setup table in Supplementary Fig. S7), DA-SOS caused phosphorylation of a downstream target of Ras, Erk, to the same degree, as did TPA stimulation (Fig. 4C, compare lanes 2 and 3). DA-SOS in the presence of TPA further enhanced phospho-Erk (Fig. 4C, lane 4), which might be explained, although does not prove, by their different mechanisms of action: DA-SOS activates Ras, whereas TPA acts downstream of Ras, adding to the activation of the pathway. DA-SOS did neither enhance spontaneous (Fig. 4C, lane 6) nor TPA-induced (Fig. 4C, lane 8) release of WtCD44 ectodomain (solCD44E). Cleavage of mutant CD44 KR-Mt barely responded to TPA treatment (Fig. 4C, compare lanes 9 and 11) and this block could not be overcome by DA-SOS (lanes 10 and 12). The column diagram shows a quantitation of 3 independent experiments. Finally, an inhibitor of MEK, a downstream effector of Ras, blocked Erk phosphorylation but did not affect CD44 cleavage (Fig. 5A). We therefore conclude that CD44 cleavage is independent of an active Ras–Erk signaling pathway.

Given that the SOS-Ras activating function of ERM proteins was not required for CD44 ectodomain cleavage, we explored other putative options. To this end, we exploited transfections with ezrin mutants. Overexpression of ezrin mutants should compete out endogenous ERM proteins. This is possible because in our cultured cells, all 3 ERM proteins are redundant with respect to Ras activation and this redundancy can be overcome by overexpression of an active mutant of one of the ERM proteins. We therefore tested
whether overexpressed ezrin mutants could reveal the function of ERM proteins that was required for CD44 cleavage. Figure 5B shows the effect of overexpressed ezrin wt or ezrin mutants. In the absence of TPA stimulation, we did not see CD44 cleavage irrespective of transfected ezrin constructs (as detected by cleavage product CD44AE; lanes 1–5; Fig. 5B). TPA-induced cleavage is shown in lanes 6 to 10. Neither wild-type ezrin (Fig. 5B, compare lanes 6 and 7) nor a phospho-mimicking mutant T567D (Fig. 5B, lane 8) affected CD44 cleavage, suggesting that endogenous ERM proteins are so abundant that additional transfected ezrin constructs made no difference. Somewhat surprisingly, however, the “inactive” ezrin T567A mutant inhibited CD44 cleavage (Fig. 5B, lane 9) suggesting that it competed with the endogenous ERM proteins for a cleavage regulatory component.

A possible lead toward this putative component and the cleavage regulatory function of ezrin was generated by the effect of the ezrin mutant R579A which cannot interact with F-actin (27). This mutant inhibited CD44 cleavage (Fig. 5B, lane 10), highlighting the possible need for an actin link to achieve CD44 cleavage. Interestingly, in respect to its inability to bind to actin, ezrin R579A mimics the cleavage inhibitory merlin whose C-terminus also cannot interact with F-actin (28, 29). These observations propose that disrupting F-actin would exert a similar inhibitory effect. We tested this assumption by adding an increasing amount of latrunculin, an inhibitor known to block actin polymerization (30). Short-term treatment (30 minutes) of the amount of latrunculin, an inhibitor known to block actin polymerization (30). Short-term treatment (30 minutes) of the

Physiologic stimuli induce ectodomain cleavage in different normal and cancer cells

TPA mimics a signaling process that is activated by numerous physiologic stimuli. Therefore, such stimuli should also result in ectodomain cleavage. This is indeed the case: in HEK293T cells that stably overexpress the angiotensin receptor (HEKNE in Fig. 5D), angiotensin II strongly induced neuregulin release. In the triple-negative breast cancer cell line MDA-MB-231, we were able to induce either endogenous or transfected CD44 cleavage by serum (see below in Fig. 7B and C), HGF, PDGF, LPA and, moderately, by FGF and EGF (Fig. 6A and B). MDA-MB-231 cells were also responsive to TPA. TPA- or HGF-induced cleavage of endogenous CD44 or overexpressed CD44 was inhibited by expression of constitutively active merlin (S518A; Fig. 6B). CD44 cleavage was also induced in MEF cells (see below: Fig. 7A). We conclude that the pathway regulating CD44 cleavage is addressed by many extracellular stimuli.

CD44 cleavage is required for cellular migration

According to our data, CD44 cleavage is a property of proliferating cells. This property as well as its blockade by the tumor suppressor NF2 (contact inhibition) should also be relevant for the control of cancer cells. We therefore explored whether CD44 cleavage was needed for cancer-relevant cellular phenotypes related to their proliferative capabilities: mobility and migration. To this end, we subjected MEF and MDA-MB-231 cells grown in confluent monolayers to scratch assays and measured their ability to close the scratch wound. After plating and scratching, cells were supplied with FCS that contains factors like LPA that we have shown to induce ectodomain cleavage. Using MEFs from mice with floxed cd44 alleles, we could compare cells expressing
CD44 with CD44-null cells (after CRE-dependent excision) and we also could substitute the cells with CD44 mutants. Supplementary Figure S6 shows photographic examples of the original scratch assay. The left picture of each condition tested shows the scratch at time 0 and the original cell number as indicated in the square. The right picture of each condition tested shows the same scratch wound after 24 hours and the percentage of the wound area still open. Quantitation of 3 experiments has been compiled in Fig. 7A. Treatment with the ADAM inhibitor batimastat strongly inhibited wound closure of migration-competent cells. CD44 plus cells (cd44<sup>+/−</sup>/C0 after CRE induction, see Supplementary Materials and Methods and Supplementary Fig. S5) repaired the scratch wound efficiently (remaining wound area 21% after 24 hours, Supplementary Fig. S6, panel 1, and Fig. 7A). Downregulation of merlin (NF2) by stably integrated shRNA enhanced the migration (0% wound remaining, Supplementary Fig. S6, panel 2, >2% in the quantitation of Fig. 7A). There was almost no wound closure by CD44-null cells (CD44<sup>−/−</sup> cells). There was almost no wound closure by CD44-null cells (CD44<sup>−/−</sup> after CRE induction, see Supplementary Materials and Methods and Supplementary Fig. S5). In these cells, NF2 was also downregulated (Supplementary Fig. S6, panel 3, and Fig. 7A). Although cd44<sup>−/−</sup>/nf2<sup>−/−</sup> was not obtained, the data suggest that NF2 did not address a pathway other than CD44. However, re-introducing CD44 wt partly re-established migration (remaining wound area 30% after 24 hours in the absence of merlin, Supplementary Fig. S6, panel 4, and Fig. 7A). This partial rescue might be explained by the fact that CD44 wt cDNA overexpression does not generate certain splice variants of CD44 that would be expressed under physiologic conditions but are missing in CD44<sup>−/−</sup> cells. Most importantly, stable transfection of the
noncleavable mutant CD44-KR-Mt as well as of a CD44 mutant that lacks the stalk region including the ADAM cleavage site could not rescue the wound-healing deficiency of CD44-null MEFs (Supplementary Fig. S6, panels 5 and 6, and Fig. 7A). These results clearly indicate that CD44 cleavage is required for a cancer relevant phenotype, cellular migration and that this function is negatively controlled by merlin.

MDA-MB-231 cells closed the scratch wound faster than MEFs. At 24 hours after scratching, wounds were totally closed (note that wound area normalized to the 0 time point; Fig. 7B). Expression of active merlin (NF2S518A) inhibited wound healing. Inactive merlin (NF2S518D) had less effect (Fig. 7B and C). Interestingly, spontaneous migration was enhanced and the inhibition by merlin rescued by expression of solCD44E (Fig. 7C) suggesting a role of CD44 ectodomain cleavage in migration.

**Discussion**

The activity state of both merlin and ERM proteins is controlled by signaling pathways that address specific protein kinases (e.g., PAK) and phosphatases (e.g., PP1). A hyaluronan–CD44–dependent signaling pathway (or cell–cell contact) favors dephosphorylation of ERM proteins and merlin, deactivating ERM proteins and activating merlin, thus establishing tumor suppression capabilities. Growth factor stimulation, in turn, activates ERM proteins and inactivates merlin, favoring growth and tumor development. In this context, our results on CD44 cleavage inhibition by merlin permit the following conclusions:

- Regulation by merlin represents an example of substrate-selective cleavage regulation. NRG1 and APP, in our cells studied, are subject to substrate-specific regulation different from CD44.
- CD44 cleavage appears to serve a tumor-promoting process by enhancing proliferation/migration of cells, including cancer cells.

TPA-induced CD44 shedding from the cell surface is regulated by ERM proteins and merlin, in contrast to other ADAM substrates, such as NRG1, c-Met, and APP. Interestingly, in neural cells, purinergic P2×7 receptor–induced APP cleavage required ERM proteins (31). Rather than direct interaction of the APP ICD with ERM proteins, this event required downstream signaling induced by ERM proteins. However, ERM activation was not always associated with the induction of APP shedding. Nerve growth factor (NGF) and benzoylbenzoyl ATP triggered ERM phosphorylation, but only the latter led to APP shedding (31). Apparently, signaling pathways can diverge after ERM activation. Similar to our results with CD44, TPA-induced ζ-selectin shedding in lymphocytes was regulated by direct interaction of ERM proteins with a proximal basic amino acid region in the cytoplasmic domain of ζ-selectin (32). The important conclusion from these reports is that substrates are specifically selected for cleavage.

Does this mean we can disregard other forms of regulation? Not at all. Regulation of ADAM activity has been widely studied (33–36). As example, we have seen ADAM activation by TPA in one of our experiments, where despite constitutive cleavage of the ICD-less CD44, TPA was still able to increase cleavage to some extent (compare lanes 7 and 10, Fig. 3B). However, CD44 is, in addition, specifically selected for cleavage on the substrate level.
Importantly, regulation of ectodomain cleavage by a tumor suppressor protein has not been observed previously. It suggests that CD44 cleavage serves a tumor-promoting function. This notion is further strengthened by the documentation that CD44 cleavage participates substantially in the regulation of cellular migration. Migration is inhibited by merlin and can be rescued by soluble CD44 ectodomain (Fig. 7). A role of CD44 in cellular migration has been observed previously (15, 17, 37–42). For instance, CD44 promoted invasion of glial cell tumor cells by its ability to bind hyaluronan (37). CD44 mediated migration of pancreatic cancer cells in conjunction with MT1-MMP (38). It has been suggested that cleavage is involved by the fact that inhibition of metalloproteases reduced migration (15); conversely, coexpression of metalloprotease with CD44 enhanced migration (42). We have described elsewhere that CD44 homodimerization is a precondition for ectodomain cleavage (M. Hartmann and colleagues; submitted for publication). Expectedly, ligation by anti-CD44 antibodies induced metalloprotease-dependent CD44 ectodomain release and migration in the aggressive tumor cell line U251MG (17, 41). Ligation-induced cleavage and migration was counteracted by expression of a dominant-negative Rac mutant (17, 41), suggesting that Rac signaling preceded cleavage. Our results are compatible with these data. Merlin action on cleavage did, however, not need to interfere with Ras/Rac (see below). Expression of soluble CD44 ectodomain, however, prevented cleavage (M. Hartmann and colleagues; submitted for publication) which seems to contradict our finding that expression of soluble CD44 enhanced migration. We have no straightforward explanation for this observation. Further analyses will be needed. The influence on migration may depend on the substratum the cells are placed on, the time course of adhesion/detachment and on adhesion molecules other than CD44.

The cleavage-promoting role of ERM proteins matches their overexpression in tumors (43–45). However, we ruled out that ERM-induced cleavage regulation requires the activation of the Ras and Rac pathway by demonstrating that an ERM-independent
constitutive activation of Ras did not influence CD44 cleavage. Ezrin mutants defective in activating guanine nucleotide exchange factors, for example, R579A or T567A prevented induced cleavage (Fig. 5B and data not shown). On the basis of the dominant-negative effect of the ezrin actin-link mutant R579A and our results using actin-disrupting latrunculin, we hypothesize that a CD44 F-actin link plays a role in the induction of its proteolysis. We have shown previously that short-term treatment with latrunculin causes only highly specific pathway disruptions (46). What the F-actin link might contribute is currently speculative. Does it support the assembly of CD44 and its protease ADAM10 in the plane of the plasma membrane? Interestingly, neuregulin release was also sensitive to an actin poison. We do however not know whether and how NRG1 is linked to F-actin.

Another intriguing observation has been reported: the induction of CD44 cleavage by treating cells with hyaluronate oligosaccharides (16). Low-molecular-weight hyaluronan does not cause activation of merlin whereas high-molecular-weight hyaluronan does (1, 3). We assume that the oligosaccharides activate cleavage-inducing signaling pathways in a CD44-independent manner (47). The oligosaccharides induce metalloprotease expression in the absence of CD44 (47). Also, the hyaluronate receptor TLR-4 (48) may cause the phenotype observed.

How does merlin interfere with CD44 cleavage? If ERM-induced Ras activity is not needed for cleavage regulation, one could assume that merlin also does not act through its Ras-inhibiting function. Our merlin mutant data indeed prove this to be correct. Ras inhibition by merlin requires that the protein is inhibiting function. Our merlin mutant data indeed prove this could assume that merlin also does not act through its Ras-receptor TLR-4 (48) may cause the phenotype observed.

Another open and highly interesting question concerns the role of CD44 cleavage in vivo, particularly in cancer. One might expect that tumors shed CD44 ectodomain at an increased rate and via this mechanism not only generate CD44 ICD, which drives the expression of proliferation-inducing genes in the nucleus, but also that cleavage products of the CD44 ectodomain might exert additional defined roles in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Hartmann, L.M. Parra, A. Ruschel, Y. Li, P. Herrlich

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