Different ADAMs have distinct influences on Kit ligand processing: phorbol-ester-stimulated ectodomain shedding of Kitl1 by ADAM17 is reduced by ADAM19

Nobuko Kawaguchi[1], Keisuke Horiuchi[2], J. David Becherer[3], Yoshiaki Toyama[2], Peter Besmer[1,*] and Carl P. Blobel[4,5,*]

[1]Developmental Biology Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA
[2]Department of Orthopedic Surgery, Keio University, School of Medicine, Tokyo 160-8582, Japan
[3]Department of Biochemical and Analytical Pharmacology, GlaxoSmithKline Inc., Research Triangle Park, NC 27709, USA
[4]Arthritis and Tissue Degeneration Program, Hospital for Special Surgery, New York, NY 10021, USA
[5]Departments of Medicine and of Physiology and Biophysics, Weill Medical College of Cornell University, New York, NY 10021, USA
*Authors for correspondence (e-mail: p-besmer@ski.mskcc.org; blobelc@hss.edu)

Accepted 10 January 2007
Journal of Cell Science 120, 943-952 Published by The Company of Biologists 2007
doi:10.1242/jcs.03403

Summary

Kit ligand (Kitl), the ligand for the Kit receptor tyrosine kinase, plays important roles in hematopoiesis, gametogenesis and melanogenesis. Kitl is synthesized as a membrane-anchored precursor that can be processed to produce the soluble growth factor. Here, we evaluated the role of ADAM (a disintegrin and metalloprotease) metalloproteases in ectodomain shedding of Kitl. We found that both ADAM17 and ADAM19 affect Kitl1 shedding, albeit in different ways. Overexpression of ADAM19 resulted in decreased levels of Endo-H-resistant mature Kitl1, thereby reducing the amount of Kitl that is shed from cells following stimulation with phorbol esters. ADAM17 was identified as the major phorbol-ester-stimulated sheddase of Kitl1, whereas ADAMs 8, 9, 10, 12 and 15 were not required for this process. ADAM17 also emerged as the major constitutive and phorbol-ester-stimulated sheddase of Kitl2 in mouse embryonic fibroblasts. Mutagenesis of the juxtamembrane domain of Kitl2 showed no stringent sequence requirement for cleavage by ADAM17, although two nonadjacent stretches of four amino acid residues were identified that are required for Kitl2 shedding. Taken together, this study identifies a novel sheddase, ADAM17, for Kitl1 and Kitl2, and demonstrates that ADAM19 can reduce ADAM17-dependent phorbol-ester-stimulated Kitl1 ectodomain shedding.

Key words: Kit ligand, ADAM17, ADAM19, Ectodomain shedding

Introduction

Proteolytic processing of transmembrane proteins to release their soluble extracellular domains from the membrane, a process termed ectodomain shedding, is emerging as an important post-translational mechanism to regulate the function of membrane proteins. A variety of structurally and functionally distinct cell surface proteins are subjected to protein ectodomain shedding, including cytokines such as tumor necrosis factor-α (TNFα), growth factors such as transforming growth factor-α (TGFα) and epidermal growth factor (EGF), receptors such as the tumor necrosis factor receptor (TNFR) 1 and TNFR2 and Notch, and adhesion molecules such as L-selectin (reviewed by Blobel, 2005; Hooper et al., 1997; Schlöndorff and Blobel, 1999; Seals and Courtneidge, 2003). The functional consequences of ectodomain shedding are diverse and include regulation of availability or active range of membrane-bound growth factors and the activation or inactivation of their receptors.

The membrane growth factor Kit ligand (Kitl), also referred to as stem cell factor (SCF), and its tyrosine kinase receptor Kit are encoded at the mouse *White spotting* (*W*) and *Steel* (*Sl*) loci, respectively (Chabot et al., 1988; Copeland et al., 1990; Geissler et al., 1988; Huang et al., 1990; Zsebo et al., 1990). Analyses of phenotypes of mutant mice have shown that the Kit receptor tyrosine kinase functions in distinct cell populations during embryonic development and in hematopoiesis, gametogenesis, melanogenesis in the postnatal animal and in interstitial cells of Cajal in the intestinal tract (Russell, 1979; Silvers, 1979). Kitl binds to its cognate receptor Kit leading to receptor dimerization, activation of its kinase activity, autophosphorylation and activation of various signaling cascades leading to cell proliferation, differentiation, migration and survival (Blume-Jensen et al., 1991; Lev et al., 1992). Although Kitl was originally identified as a soluble protein (Nocka et al., 1990), Kitl is synthesized as a transmembrane protein that may be processed to produce soluble Kitl (Huang et al., 1990). Alternative splicing generates two Kitl RNA transcripts that encode two cell-associated Kitl proteins, designated Kitl1 and Kitl2 (Flanagan et al., 1991; Huang et al., 1992). Whereas the Kitl1 protein is rapidly cleaved at a major cleavage site located in exon 6, releasing a soluble protein, by contrast the Kitl2 protein lacks the exon 6 sequences and is processed less efficiently (Huang et al., 1992). Although both membrane-bound and soluble Kitl appear to be biologically active in vitro, the phenotypes of mice carrying the Sl^d allele, which produces only a secreted soluble Kitl protein as a result of an intragenic deletion, imply that membrane Kitl and presumably juxtacrine signaling are crucial

for normal function in vivo (Brannan et al., 1991; Flanagan et al., 1991). In agreement with this conjecture, mice that express Kitl2 exclusively have only minor deficiencies in hematopoiesis and mast cell development (Tajima et al., 1998).

The mechanisms responsible for Kitl processing are poorly understood. Early studies of Kitl processing showed that the production of soluble Kitl in COS cells is inducible and may be regulated by the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA) (Huang et al., 1992). Studies using different protease inhibitors implied that Kitl1 and Kitl2 are processed by distinct proteolytic activities (Pandiella et al., 1992). Some proteases, including matrix metalloproteinase (MMP) 9 (Heissig et al., 2002), mast cell chymase (chymotrypsin-like protease) (Longley et al., 1997) and several members of the ADAMs (a disintegrin and metalloprotease) family of metallopeptidases (Amour et al., 2002; Chesneau et al., 2003; Mohan et al., 2002; Roghani et al., 1999; Zou et al., 2004) (Table 1) have been suggested to have activities that mediate Kitl shedding. Thus, tissue expression of differentially spliced mRNAs encoding Kitl1 and Kitl2 and the tissue-specific expression of proteolytic activities capable of processing Kitl to produce soluble Kitl may provide mechanisms for the regulation of Kitl function.

ADAMs are a large family of transmembrane metallopeptidases that have crucial roles in ectodomain shedding (for reviews, see Blobel, 2005; Seals and Courtneidge, 2003; White, 2003). ADAMs consist of an N-terminal signal sequence followed by a pro domain, metalloprotease domain, disintegrin domain, cysteine-rich domain, epidermal growth factor-like domain, transmembrane domain and cytoplasmic domain. ADAMs have been implicated in diverse functions, including fertilization (Primakoff and Myles, 2000), neurogenesis (Fambrough et al., 1996; Hattori et al., 2000), angiogenesis (Hartmann et al., 2002; Horiuchi et al., 2003), adipogenesis (Kawaguchi et al., 2003; Kawaguchi et al., 2002), heart development (Horiuchi et al., 2005; Jackson et al., 2003; Kurohara et al., 2004; Zhou et al., 2004), asthma (Van Eerdewegh et al., 2002) and cancer (Kveiborg et al., 2005; Peduto et al., 2005). A conserved zinc-binding catalytic site sequence motif (HEXXH) (Stocker et al., 1995) is found in approximately half of the known ADAMs. Of these, ADAM17 (also referred to as tumor necrosis factor-α converting enzyme, TACE) is one of the best-characterized members of this gene family and is known to be required for PMA-stimulated ectodomain shedding of a broad number of transmembrane protein substrates, including the pro-inflammatory cytokine TNFα (Black et al., 1997; Moss et al., 1997; Zheng et al., 2004) and the EGF-receptor (EGFR) ligands amphiregulin, heparin-binding EGF-like growth factor (HB-EGF) and TGFα (Merlos-Suarez et al., 2001; Peschon et al., 1998; Sahin et al., 2004; Sternlicht et al., 2005; Sunnarborg et al., 2002). Mice lacking ADAM17 die shortly after birth, most probably because of defects in the functional activation of the EGFR ligands (Jackson et al., 2003; Peschon et al., 1998; Sternlicht et al., 2005). Recently, ADAM17 was shown to mediate the shedding of the Kit receptor tyrosine kinase ectodomain, the only known receptor for Kitl (Cruz et al., 2004).

In this study, we evaluated the contribution of catalytically active ADAMs to Kitl shedding. Although previous studies that tested whether purified ADAMs can cleave Kitl1 peptides covering the major cleavage site revealed a possible role of several ADAMs in Kitl1 shedding (Amour et al., 2002; Chesneau et al., 2003; Mohan et al., 2002; Roghani et al., 1999; Schlomann et al., 2002) (see Table 1), the actual role of those ADAMs in Kitl shedding in cells or in vivo has not yet been evaluated. To address this issue, we isolated primary mouse embryonic fibroblasts (mEFs) from mice that are deficient in several widely expressed, catalytically active ADAMs (8, 9, 10, 12, 15, 17 and 19), and evaluated how the loss of these ADAMs affects Kitl1 as well as Kitl2 shedding. Moreover, we evaluated the mechanism underlying the previously observed function of ADAM19 as a negative regulator of PMA-stimulated Kitl1 shedding (Chesneau et al., 2003). Our results demonstrated that both ADAM17 and ADAM19 affect Kitl1 processing in cell-based assays, and identified ADAM17 as the major sheddase for Kitl2. Interestingly, whereas processing by ADAM17 resulted in ectodomain shedding of Kitl1 from cells, overexpression of ADAM19 decreased PMA-stimulated Kitl1 ectodomain shedding by reducing the levels of mature Kitl1 available for release, thus implicating an ADAM in a processing event that decreases stimulated release of a membrane-anchored substrate.

Table 1. Kitl1 peptide cleavage sites used by various ADAMs in vitro

ADAMs	Kitl1 peptide cleavage sites	References
ADAM8	PPVA**A↓S**SLRN	(Amour et al., 2002)
ADAM9	PPV**A↓A↓S↓S**LRN	(Roghani et al., 1999)
ADAM10	No cleavage	(Amour et al., 2002)
ADAM17	PPVA**A↓S**SLRN	(Mohan et al., 2002)
ADAM19	PPVA**A↓S**SLRN	(Chesneau et al., 2003)
ADAM33	PPVA**A↓S**SLRN	(Zou et al., 2004)

Results

ADAM19 reduces stimulated processing of Kitl1

The ability of ADAM19 to cleave Kitl1 peptides in vitro (Table 1) prompted us to evaluate the potential role of ADAM19 in shedding of Kitl1 in cell-based assays. To increase the sensitivity of detection of the precursor and shed forms, we used expression plasmids encoding full-length Kitl1 fused to an alkaline phosphatase (AP) module at the N-terminus, such that the AP module is present in the extracellular domain of Kitl1 and was released into the culture medium after shedding. Since a previous study suggested that overexpression of ADAM19 strongly reduced the PMA-stimulated shedding of Kitl1 (Chesneau et al., 2003), and because a robust response to short treatment with PMA (30-60 minutes) is considered a hallmark property of the related ADAM17 (Horiuchi et al., 2007), we first tested whether overexpression of ADAM19 also affects the shedding of TGFα, the PMA-stimulated release of which depends on ADAM17. We found that under conditions where overexpression of ADAM19 strongly reduced PMA-stimulated shedding of Kitl1 compared with the catalytically inactive E>A mutant of ADAM19 (Fig. 1A, top panel, compare lanes 2 and 4) (see also Chesneau et al., 2003), or compared with cells expressing only Kitl1 (data not shown), overexpression of ADAM19 did not affect the PMA-dependent shedding of TGFα compared with ADAM19E>A (Fig. 1A, bottom panel, compare lanes 2 and 4). These results argue against a direct effect of ADAM19 on the activity of ADAM17, and demonstrate that the catalytic activity of ADAM19 is necessary for its effect on Kitl1 shedding. When we examined

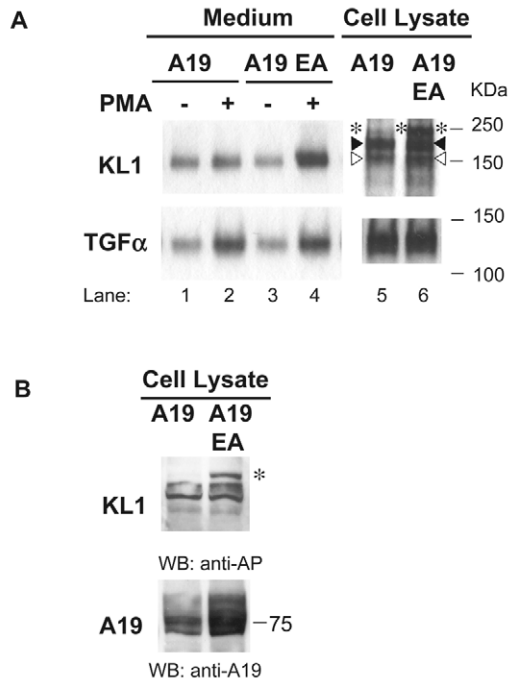

Fig. 1. Effect of overexpression of ADAM19 on Kitl1 shedding. (A) Wild-type ADAM19 (lanes 1 and 2) or ADAM19 carrying an inactivating E>A mutation in its catalytic site (A19 EA) (lanes 3 and 4) were transfected into COS-7 cells together with AP-tagged Kitl1 (upper panel) or TGFα (lower panel). Kitl1 shedding was stimulated with 25 ng/ml PMA as described in the Materials and Methods. Briefly, medium was collected before and after addition of PMA in the same well for 1 hour and analyzed on 8% SDS-PAGE. Comparison of cell lysates expressing Kitl1 or TGFα and either ADAM19 or the E>A mutant showed similar expression levels of pro-TGFα and two forms of Kitl1 (black and open arrowhead), whereas much less of the slowest migrating form of Kitl1 (indicated by an asterisk) was seen in cells expressing ADAM19 compared with cells expressing the E>A mutant. (B) Western-blot analysis of the expression of Kitl1 (upper panel) and ADAM19 or ADAM19 E>A (lower panel) in COS-7 cells. The slowest migrating form of Kitl1, which is not present in the sample overexpressing ADAM19, is indicated by an asterisk.

the lysates of cells expressing Kitl1 together with either ADAM19 or ADAM19E>A, we found similar expression levels of a 200 kDa pro-form of Kitl1. However, the levels of a slower migrating form of Kitl1 of 240 kDa were strongly reduced in cells expressing ADAM19 compared with controls expressing the inactive ADAM19E>A mutant (Fig. 1A, top panel, lanes 5 and 6, and Fig. 1B). The expression of pro-TGFα was comparable in cells expressing wild-type or E>A mutant ADAM19 (Fig. 1A, lower panel, lanes 5 and 6). Essentially identical results were obtained when these experiments were repeated in Chinese hamster ovary (CHO) cells (data not shown).

ADAM17, but not ADAM8, 9, 10, 12 or 15 is required for stimulated Kitl1 shedding in mEFs

The ability of several purified catalytically active ADAMs to cleave a peptide corresponding to the major constitutive cleavage site of Kitl1 in vitro (shown in Table 1) (Amour et al., 2002; Chesneau et al., 2003; Mohan et al., 2002; Roghani et al., 1999; Schlomann et al., 2002) raised the possibility that members of the ADAM family of membrane-anchored metallopeptidases function as Kitl sheddases. To test this hypothesis, we performed Kitl1 shedding assays in primary mEFs derived from different ADAM-deficient mice at embryonic day E13.5 [*Adam8*$^{-/-}$, *Adam9/12/15*$^{-/-}$ (homozygous mutant form of *Adam9*, *Adam12* and *Adam15*), *Adam17*$^{-/-}$ and wild-type controls]. In the case of *Adam10*$^{-/-}$ and *Adam10*$^{+/-}$ cells, only immortalized mEFs could be used since *Adam10*$^{-/-}$ mice do not survive past E9.5 (Hartmann et al., 2002). As seen in Fig. 2A, shedding of Kitl1 from wild-type mEFs can be stimulated by addition of PMA or pervanadate (PV) for 1 hour. Both PMA- and PV-stimulated shedding in *Adam8*$^{-/-}$, *Adam9/12/15*$^{-/-}$ and *Adam10*$^{-/-}$ mEFs was similar to that seen in the corresponding wild-type control mEFs. Even though there was some variability in the level of stimulation by PMA or PV from experiment to experiment, the control cells that were analyzed at the same time together with *Adam8*$^{-/-}$ or *Adam9/12/15*$^{-/-}$ or *Adam10*$^{-/-}$ cells always displayed a comparable level of stimulation as the *Adam*$^{-/-}$ cells (Fig. 2A). However, the PMA-dependent increase in Kitl1 shedding was abolished in *Adam17*$^{-/-}$ mEFs, and the response to PV was significantly reduced (Fig. 2A, bottom panel, lanes 3, 4, 7 and 8). These results provide the first evidence for an essential role of ADAM17 in PMA- and PV-stimulated Kitl1 shedding in mEFs. The BB94-dependent reduction of Kitl1 shedding was not affected in any of the *Adam*$^{-/-}$ mEFs tested here compared with wild-type controls, arguing against a role for ADAMs 8, 9, 10, 12, 15, 17 and 19 in constitutive BB94-sensitive Kitl1 shedding. Finally, PMA- and PV-stimulated shedding of Kitl1 in *Adam17*$^{-/-}$ cells could be restored by reintroduction of wild-type mouse ADAM17 (Fig. 2B, PV-stimulated shedding not shown).

Overexpression of ADAM19 decreases levels of mature Kitl1

The results shown in Fig. 2 demonstrate that PMA- and PV-stimulated shedding of Kitl1 depend on ADAM17. However, since overexpression of ADAM19 did not affect the stimulated shedding of TGFα, which also depends on ADAM17, this suggested that the effect of overexpressed ADAM19 on Kitl1 shedding is not a consequence of inactivation of ADAM17. To gain further insights into the mechanism underlying the decrease in PMA- and PV-stimulated Kitl1 shedding in cells overexpressing ADAM19, we performed a pulse-chase analysis to determine whether ADAM19 might affect intracellular maturation or processing of Kitl1. COS-7 cells were transfected with Kitl1 alone, or Kitl1 with either wild-type ADAM19 or the ADAM19E>A mutant, then labeled with [^{35}S]-methionine for 30 minutes and chased for different time periods as indicated in Fig. 3. The cell lysates were collected and immunoprecipitated with antibodies against mouse Kitl. In cells expressing only Kitl1 and in cells coexpressing catalytically inactive ADAM19E>A or wild-type ADAM19 together with Kitl1, similar amounts of core-glycosylated forms of Kitl1 were visible at all time points throughout the chase period (Fig. 3, lanes 1–4, indicated by asterisks). However, after 1, 3 and 6 hours of chase, very little of the slowest migrating form of Kitl1, indicated by an black arrow in Fig. 3, was visible in cells coexpressing wild-type ADAM19,

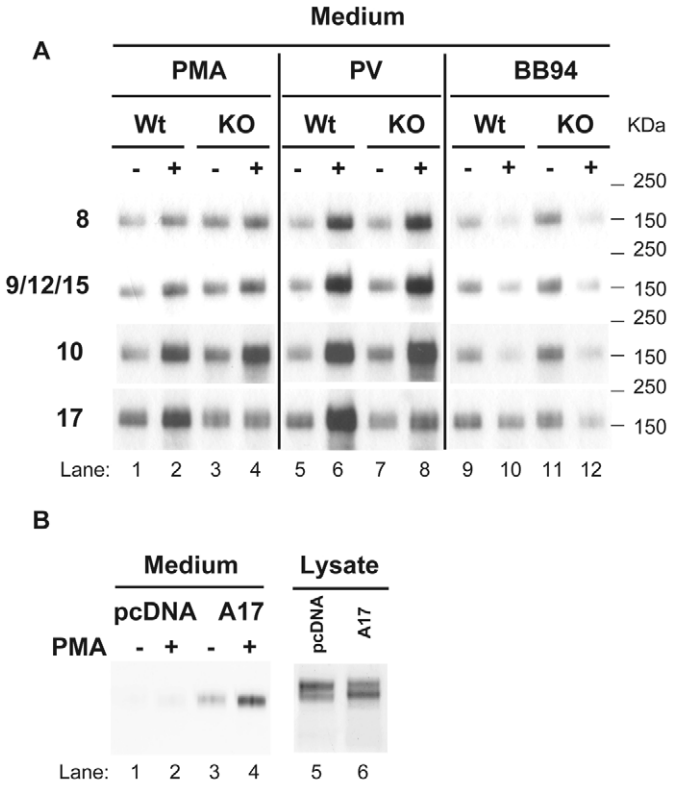

Fig. 2. ADAM17 is required for PMA- and PV-stimulated shedding of Kitl1 in mEFs, whereas ADAMs 8, 9, 10, 12 and 15 are dispensable. (A) Kitl1 shedding was analyzed in primary mEFs isolated from E13.5 wild-type controls (lanes 1, 2, 5, 6, 9 and 10) or from corresponding *Adam8*$^{-/-}$, *Adam9/12/15*$^{-/-}$, *Adam17*$^{-/-}$ embryos (lanes 3, 4, 7, 8, 11 and 12). *Adam10*$^{-/-}$ cells (lanes 3, 4, 7, 8, 11 and 12) and *Adam10*$^{+/-}$ controls (shown under wild type in lanes 1, 2, 5, 6, 9 and 10) were immortalized since *Adam10*$^{-/-}$ embryos die at E9.5. Whereas PMA- and PV-induced shedding was comparable between *Adam8*$^{-/-}$ and *Adam9/12/15*$^{-/-}$ mEFs and cells isolated from their respective wild-type controls, as well as between *Adam10*$^{-/-}$ and *Adam10*$^{+/-}$ cells, PMA-dependent increase in shedding of Kitl1 was abolished (lane 4) and PV-induced shedding was significantly reduced in *Adam17*$^{-/-}$ cells (lane 8) compared with cells from wild-type controls (lanes 2 and 6). In all *Adam*$^{-/-}$ and wild-type cells analyzed here, the BB94-sensitive component of constitutive Kitl1 shedding was unaffected (lanes 10 and 12 compared with lanes 9 and 11, respectively), suggesting that other metalloproteases besides the ADAMs tested here are responsible for constitutive Kitl1 shedding. (B) PMA-stimulated Kitl1 shedding in *Adam17*$^{-/-}$ cells (lane 2) could be rescued by coexpression of mouse ADAM17 (lane 4). A western blot of the cell lysates of *Adam17*$^{-/-}$ cells expressing Kitl1 (lane 5) or Kitl1 and ADAM17 (lane 6) is shown on the right. Overexpression of ADAM17 reduces the slowest migrating form of Kitl1 in *Adam17*$^{-/-}$ cells. All experiments were repeated four times with essentially identical results.

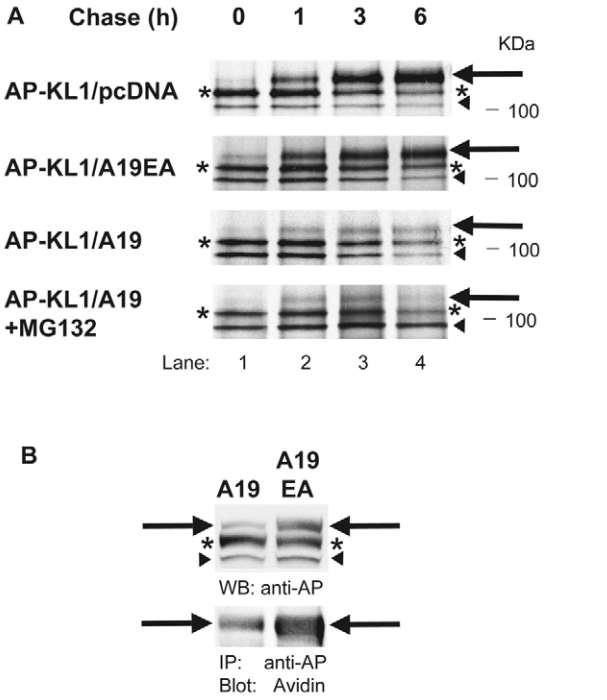

Fig. 3. Effect of overexpressed ADAM19 on levels of mature Kitl1. (A) COS-7 cells were transfected with AP-tagged Kitl1 (top panel), or with Kitl1 together with either the catalytically inactive ADAM19E>A mutant (second panel from the top), or wild-type ADAM19 (third panel from the top), or wild-type ADAM19 in the presence of the proteasome inhibitor MG132 (lower panel) labeled with [^{35}S]-methionine for 30 minutes, and chased in OPTI-MEM medium. The cell lysates were collected at the indicated time points, immunoprecipitated with anti-Kitl antibody, and the immunoprecipitated material was separated by 8% SDS-PAGE and visualized by autoradiography. A black arrow indicates the mature form of Kitl1 (125 KDa), an asterisk is next to the intermediate form I (112.5 KDa) and a black arrowhead points to the intermediate form II (100 KDa). It should be noted that immunoprecipitated AP-tagged Kitl1 runs as a monomer on SDS-PAGE since it is reduced and boiled in SDS sample buffer. Therefore, it has a different mass than in the in-gel AP-assay shown in Fig. 1A, where AP-tagged Kitl1 runs as a dimer since the material is not boiled before SDS-PAGE. (B) Effect of ADAM19 on Kitl1 that can be biotinylated on the cell surface. Kitl1 was expressed in COS-7 cells together with either ADAM19 or ADAM19E>A, and cell surface proteins were labeled with a non-membrane-permeable biotinylation reagent as described in the Materials and Methods. The upper panel shows a western blot of Kitl1 in cell lysates, and the lower panel shows cell-surface biotinylated material that was immunoprecipitated with an antibody against the alkaline phosphatase moiety attached to Kitl1, transferred to nitrocellulose and probed with HRP-labeled streptavidin (avidin). The amount of Kitl1 that can be cell-surface biotinylated is reduced in the presence of ADAM19.

whereas much higher levels of this mature form of Kitl1 accumulated in cells transfected with the ADAM19E>A mutant and in cells expressing only Kitl1. To assess whether ADAM19 reduces the amount of Kitl1 at the cell surface, we used a membrane-impermeable biotinylation reagent to label proteins on the surface of cells expressing Kitl1 and ADAM19 or ADAM19E>A. Kitl1 was immunoprecipitated with an antibody against its AP-tag, transferred to nitrocellulose and biotinylated material was detected with horseradish peroxidase (HRP)-labeled streptavidin. This corroborated that overexpression of ADAM19 reduces the amount of Kitl1 on the cell surface (Fig. 3B, lower panel), but does not reduce the core-glycosylated form of Kitl1 detected by western-blot analysis with an anti-AP antibody (Fig. 3B, upper panel,

marked by asterisk). The proteasome inhibitor MG132 did not block the effect of wild-type ADAM19 on appearance of mature pro-Kitl1, arguing against a role of proteasomal degradation in this process (Fig. 3A, lower panel).

ADAM17 is required for stimulated shedding of Kitl2

Kitl2 is a splice variant of Kitl lacking exon 6, which contains the major physiological cleavage site of Kitl1 (Fig. 4A). Kitl2 is therefore more likely to remain as a membrane-associated form. When we investigated whether ADAM19 might also affect Kitl2 shedding, we found no evidence for an effect of overexpressed ADAM19 on PMA-stimulated shedding of Kitl2 compared with cells co-transfected with the ADAM19E>A mutant or cells expressing only Kitl2 (Fig. 4B). Thus, ADAM19 only affects the shedding of Kitl1, but not that of Kitl2. However, in *Adam17*$^{-/-}$ cells, both PMA- and PV-stimulated shedding of Kitl2 were almost completely abolished (Fig. 4C, PMA data not shown). Overexpression of mouse ADAM17 rescued constitutive and stimulated shedding in *Adam17*$^{-/-}$ cells (Fig. 4C), confirming that the defect of shedding in *Adam17*$^{-/-}$ cells is because of the loss of ADAM17. This result demonstrates that ADAM17 is the major constitutive and PMA-stimulated sheddase for Kitl2 in mEF cells.

Evaluation of the effect of deletions on Kitl shedding

Whereas soluble Kitl forms non-covalent homodimers, each Kitl monomer contains two intra-chain disulfide bonds, C4-C89 and C43-C138 (Arakawa et al., 1991; Jiang et al., 2000; Langley et al., 1992; Zhang et al., 2000). The Kitl$^{1\text{-}141}$ sequences represent the functional core of Kitl that contains the dimer interface and the domain involved in Kit receptor binding and are sufficient to mediate cell proliferation activity (Langley et al., 1994). In addition, previous reports have identified a major cleavage site at A164 or A165 (Zsebo et al., 1990), and two additional minor secondary cleavage sites near S142 and KAAK177-180 (Cheng and Flanagan, 1994; Majumdar et al., 1994). In the light of these observations, we hypothesized that the cleavage site for ADAM17 in Kitl must be located C-terminally of C138, and thus potentially around the boundary region of exon 6. In order to test this hypothesis, we prepared eight deletion mutants in this region (Fig. 5A) and evaluated their effect on Kitl shedding.

In order to rule out that these deletions cause improper protein folding, which would result in retention by endoplasmic reticulum (ER) chaperones and intracellular degradation, we used acquisition of resistance to endodeglycosidase H (Endo H) as an indicator that the expressed mutant glycoproteins were able to fold, had been released from the ER and had traversed the medial Golgi complex. If a mutant did not acquire resistance to Endo H, this would suggest that the mutation prevented proper protein folding, resulting in retention in the ER. Since an unfolded mutant protein would be unable to encounter a functional sheddase such as ADAM17, which only becomes active after its pro-domain is removed in the trans-Golgi network, any mutation(s) that affect(s) protein folding would not be informative in terms of how the mutation influences processing by ADAM17. COS-7 cells expressing wild-type Kitl1 were metabolically labeled with [^{35}S]-methionine, chased for 6 hours and their cell lysates were either treated with Endo H or

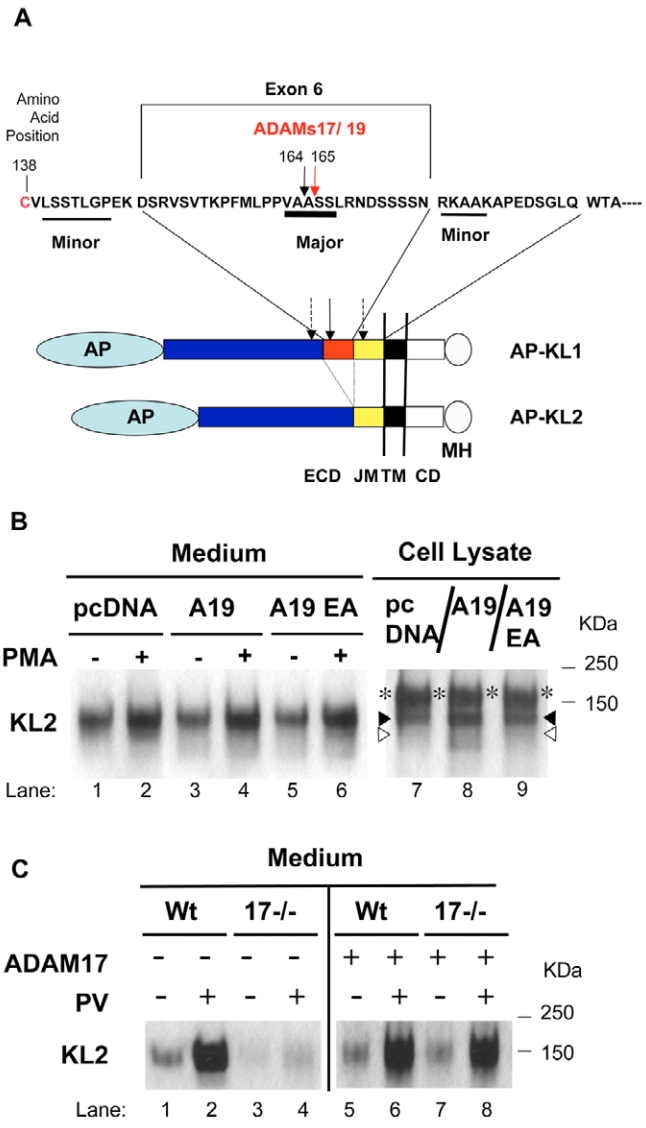

Fig. 4. Kitl2 shedding by ADAM17 and ADAM19. (A) The schematic presentation of AP-tagged wild-type Kitl1 and Kitl2. Arrows indicate the positions of the major proteolytic cleavage site at A164 and A165 in Kitl1. One of these sites, A165, corresponds to the Kitl1 cleavage site used by ADAM17 and ADAM19 in vitro [(23,26) see also Table 1]. Exon 6, which contains the putative major cleavage site, is spliced out in Kitl2. AP, alkaline phosphatase tag; CD, cytoplasmic domain; ECD, extracellular domain; JM, juxtamembrane domain; MH, Myc-His tag; TM, transmembrane domain. (B) AP-tagged Kitl2 was either expressed alone or together with wild-type ADAM19 or the catalytically inactive ADAM19E>A mutant (A19 EA) (lanes 3 and 4) in COS-7 cells, and shedding was stimulated with 25 ng/ml PMA. ADAM19 did not affect Kitl2 shedding into the medium or the levels of mature Kitl2 in cell lysates (indicated by an asterisk) compared with ADAM19E>A or cells expressing only Kitl2. (C) Kitl2 shedding in *Adam17*$^{-/-}$ and wild-type control mEFs. Both constitutive and PV-stimulated shedding of Kitl2 was strongly reduced in *Adam17*$^{-/-}$ cells (lanes 3 and 4) compared with wild-type control mEFs (lanes 1 and 2). The defect in Kitl2 shedding in *Adam17*$^{-/-}$ cells could be rescued by overexpressed wild-type mouse ADAM17 (lanes 7 and 8), confirming that ADAM17 is the major sheddase for both constitutive and PMA-stimulated shedding of Kitl2.

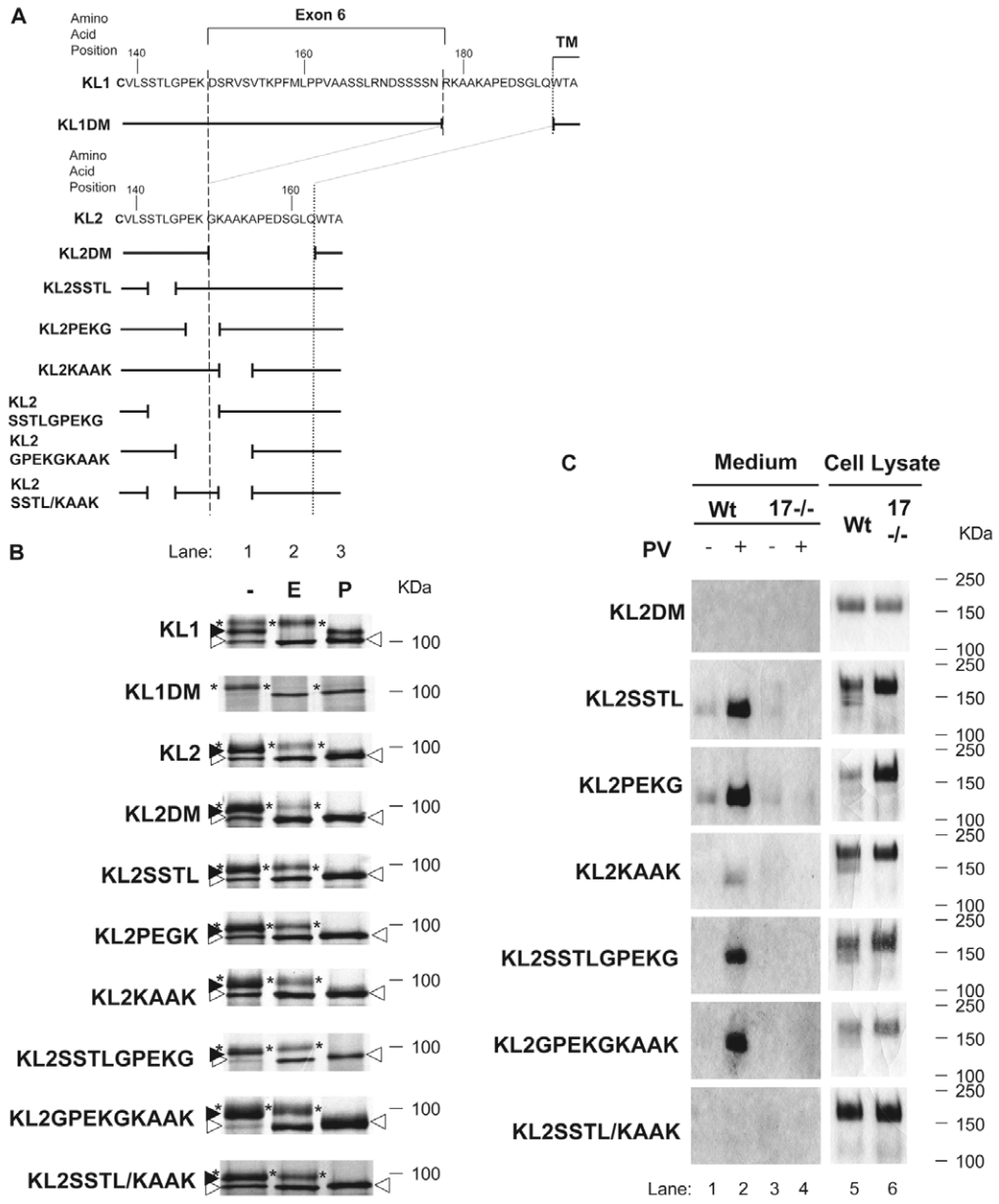

Fig. 5. Evaluation of how mutations in the juxtamembrane domain affect Kitl shedding. (A) Mutants carrying deletions in the juxtamembrane domain of Kitl1 or Kitl2 were generated as described in the Materials and Methods in order to identify the cleavage site(s) for ADAM17. The designations of individual deletion mutants are listed on the left. All expression constructs used in this study contain an N-terminal AP-tag and a C-terminal Myc-His tag. Exon 6, which contains the putative major cleavage site, is spliced out in Kitl2. TM, transmembrane domain. Two different kinds of vertical lines indicate the boundary for exons, and the boundary between the extracellular and transmembrane domain, respectively. (B) COS-7 cells were transfected with AP-tagged wild-type Kitl1, Kitl2 or various mutant plasmids, labeled with [^{35}S]-methionine for 30 minutes and chased for 6 hours. Cell lysates were collected, immunoprecipitated with anti-Kitl antibody and treated with Endo H (E, lane 2) or PNGase F (P, lane 3) at 37°C overnight, as described in the Materials and Methods. Untreated sample was loaded in lane 1. Asterisks, black arrowheads and white arrowheads indicate the mature form (125 KDa), intermediate form II (112.5 KDa) and intermediate form I (100 KDa) of Kitl, respectively. The mature forms of Kitl in cells transfected with Kitl2 mutants were resistant to treatment with Endo H to a similar degree as wild-type Kitl2. Since Endo H resistance is acquired during passage through the medial Golgi compartment, these results indicate that similar amounts of the mutant and wild-type proteins were released from the ER and migrated through the medial Golgi compartment. This strongly suggests that the mutants giving rise to Endo H-resistant mature forms are properly folded, and that the lack of shedding of KL2SSTL/KAAK is not caused by retention of this mutant in the ER. Since the KL1DM mutant did not acquire resistance to Endo H it was not used for further shedding analysis. (C) Effects of deletions on Kitl shedding. AP-tagged deletion variants were transfected in wild-type (lanes 1 and 2) or *Adam17*$^{-/-}$ mEFs (lanes 3 and 4). Shedding was performed as described above, using 100 mM PV as a stimulus. Supernatants (lanes 1-4) were concentrated by ConA-Sepharose and analyzed on 8% SDS-PAGE as described in the Materials and Methods. An AP-analysis of cell lysates shows similar expression of mutant forms of Kitl2 in wild-type and *Adam17*$^{-/-}$ cells in cases in which shedding is abolished. Lower levels of Kitl2 mutants were seen in wild-type cell lysates following PV or PMA stimulation compared with *Adam17*$^{-/-}$ cells, presumably because the mutants accumulate in the absence of ADAM17.

peptide N-glycanase F (PNGase F), which removes all N-linked carbohydrate residues, or mock treated (Fig. 5B, top panel, lanes 1–3). The slowest migrating form of Kitl1 (indicated by asterisks) was resistant to Endo H, indicating that it had passed through the medial Golgi complex, whereas the faster migrating intermediate form (indicated by the black arrowhead) was sensitive to Endo H treatment. Wild-type Kitl2 as well as all Kitl2 mutants gave rise to comparable relative levels of Endo-H-resistant forms, confirming that all Kitl2 mutants are released from the ER and can pass through the medial Golgi complex. Immunofluorescence analysis of COS-7 cells that were either permeabilized with Triton X-100 or not permeabilized showed that the staining pattern of all mutants was indistinguishable from that of wild-type Kitl2 (data not shown). The only exception was the deletion mutant of Kitl1, KL1DM, which did not acquire resistance to Endo H, suggesting that this very membrane-proximal deletion mutation prevented proper protein folding. The subsequent shedding studies therefore focused on Kitl2 and Kitl2 mutants that were not retained in the ER.

First, we tested the shedding profile of Kitl2 mutants with sequential four amino acid deletions: SSTL141-144, PEKG146-149 or KAAK150-153 (KL2SSTL, KL2PEKG and KL2KAAK). Surprisingly, none of these deletions abolished constitutive or PV-stimulated shedding (Fig. 5C). Therefore, we next tested mutants with larger deletions of eight or nine amino acid residues. Interestingly, the shedding of a mutant carrying two non-adjacent deletions of four amino acids each (SSTL141-144 and KAAK150-153; KL2STTL/KAAK) was completely abolished, whereas the shedding of two mutants containing a deletion of nine contiguous amino acid residues covering either the SSTL141-144 or KAAK150-153 sequence (KL2SSTLGPEKG or KL2GPEKGKAAK) was strongly stimulated by PV (Fig. 5C). When we tested the shedding of the mutants described above in the presence of PMA, we obtained essentially the same results as following stimulation with PV (data not shown).

Discussion

Proteolytic release of Kitl from its membrane anchor is known to have an important role in regulating juxtacrine versus paracrine functions of Kitl. This prompted us to identify and characterize novel proteases that are able to process Kitl1 as well as the splice variant Kitl2, which lacks several cleavage sites for proteolytic enzymes that can participate in ectodomain shedding of Kitl1. Our results identified two ADAMs, ADAM17 and ADAM19, as enzymes that can affect Kitl processing. We will first discuss how ADAM19 might affect the intracellular maturation or degradation of Kitl1, thereby reducing its stimulated shedding into the supernatant. Then we will discuss the identification of ADAM17 as a major phorbol-ester- and phosphatase-inhibitor-stimulated sheddase of Kitl1 and Kitl2, and will consider the implications of these results for the function of Kitl1 and Kitl2 in vivo.

Previously, we had found that overexpression of ADAM19, which is able to process the cleavage site peptide for Kitl1 in vitro, decreased the stimulated shedding of Kitl1 from cells (Chesneau et al., 2003). Since overexpression of ADAMs usually increases the release of their substrates into the cell supernatant, this result raised the possibility that ADAM19 either cleaves and inactivates the sheddase for Kitl1 or activates an inhibitor of Kitl1 shedding. In the current study, we found that increased expression of ADAM19 did not affect the release of TGFα, a substrate of ADAM17. This argues against an effect of overexpressed ADAM19 on the catalytic activity of ADAM17. Instead, we observed that overexpression of ADAM19 strongly reduced the ratio of mature membrane-associated pro-Kitl1 in cell lysates compared with immature forms of pro-Kitl1, whereas overexpression of the catalytically inactive ADAM19E>A mutant did not. These findings were inconsistent with a model in which ADAM19 releases an inhibitor of Kitl1 shedding, as this should have resulted in increased levels of mature pro-Kitl1 in cells. Finally, when we performed loss-of-function studies in mEFs lacking candidate sheddases of the ADAM protease family, we found that ADAM17 is the main enzyme responsible for phorbol-ester- and phosphatase-inhibitor-stimulated release of Kitl1.

In the light of these results, an alternative hypothesis for how overexpression of ADAM19 might decrease ADAM17-dependent PMA-stimulated shedding of Kitl1 is that ADAM19 cleaves Kitl1 intracellularly, resulting in the intracellular degradation of the ectodomain instead of its release from the cell. It can also not be ruled out that overexpression of ADAM19 leads to a slight increase in constitutive Kitl1, but such an increase might not be evident in comparison with normal amounts of constitutive Kitl1 shedding. Moreover, it is conceivable that ADAM19 somehow prevents maturation of Kitl1, although this would have to depend on both its catalytic activity and on the presence of the ADAM19 cleavage site, since inactive ADAM19 does not affect Kitl1 maturation, and active ADAM19 does not reduce shedding of Kitl2, which differs from Kitl1 only by the absence of the ADAM19 cleavage site in exon 6. Nevertheless, both possibilities are consistent with the results of a pulse-chase analysis, in which overexpressed ADAM19 led to lower levels of mature Kitl1 without affecting biosynthesis of the immature precursor. The consequence of overexpressing ADAM19 is thus a reduction of the amount of mature, Endo-H-resistant Kitl1 that is available for ADAM17-dependent PMA-stimulated shedding. Since ADAM19 can release other substrates into the supernatant when it is overexpressed, including TNFα and TNFα-related activation-induced cytokine (TRANCE), also referred to as osteoprotegerin ligand (OPGL) (Chesneau et al., 2003; Zhou et al., 2004), it will now be interesting to determine why Kitl1 shedding is not increased by overexpressed ADAM19. Perhaps overexpressed ADAM19 processes Kitl1 in a different subcellular location within the secretory pathway compared with other ADAM19 substrates. Moreover, it cannot be ruled out that ADAM19 activates another enzyme that is responsible for the intracellular degradation of Kitl1. As there is an increased release of Kitl1 from *Adam19*$^{-/-}$ cells compared with wild-type controls, endogenous levels of ADAM19 are evidently also able to influence the regulation of Kitl1 shedding from mEFs (Chesneau et al., 2003).

Although the predicted cleavage site for ADAM17 in Kitl1 is lacking in Kitl2 (Table 1), we found that ADAM17 is required for PMA- and PV-dependent release of Kitl2 from mEFs. ADAM17 must therefore use a different cleavage site in Kitl2 than the one used by purified ADAM17 in the Kitl1 cleavage site peptide in vitro. To further define the cleavage site for ADAM17 in Kitl2, we tested how mutants in the juxtamembrane domain of Kitl2 impinge on its ectodomain

shedding in cell-based assays. Although Majumdar et al. had previously reported that Kitl1 lacking the sequence KAAK178-181 together with the mutation in the major proteolytic cleavage site (VLES163-166 instead of VAAS163-166) remains membrane associated without producing soluble Kitl (Majumdar et al., 1994), the KAAK150-153 deletion in Kitl2, in which the major proteolytic cleavage site is lacking and therefore expected to have an effect as previously shown, was not enough to abolish PMA- or PV-stimulated Kitl shedding completely in our study. Only a Kitl2 mutant lacking both the KAAK150-153 and SSTL141-144 sequences as well as a mutant lacking 13 amino acid residues (G149-Q161) did not produce detectable amounts of soluble Kitl. The deletion of 13 amino acid residues in KL2DM might reduce the distance between the cleavage site and the plasma membrane to a point where ADAM17 can no longer gain access to the site. However, this is probably not the case with the KL2SSTL/KAAK mutation, in which only eight amino acid residues were deleted, since two mutants in which nine amino acid residues were deleted are shed efficiently (KL2SSTLGPEKG and KL2GPEKGKAAK). One possible explanation for the lack of processing of KL2SSTL/KAAK is that ADAM17 has an extended recognition sequence in Kitl2 for processing that includes these two sequences, but requires only one to be present. This notion is consistent with the crystal structure of ADAM17, which shows an extended peptide-binding groove in the catalytic site (Maskos et al., 1998). Finally, it is possible that ADAM17 uses binding sites in the ectodomain of Kitl as a determinant for its recognition, and then proceeds to cleave the substrate in a membrane proximal position without a strong cleavage site preference, but nevertheless requires either SSTL141-144 or KAAK150-153 to be present in Kitl2.

These studies thus define two mutants of Kitl that are produced at levels comparable to wild-type Kitl in cells and do not affect transport to the cell surface, but nevertheless abolish Kitl shedding. This information might be useful for generating knock-in mutations in mice that abolish shedding of Kitl in order to dissect the roles of soluble and membrane-associated Kitl in mouse development and disease models. Similar knock-in mutations have corroborated the essential role for soluble HB-EGF in heart valve development (Yamazaki et al., 2003), and helped dissect the contribution of membrane-anchored versus soluble TNFα in the immune response (Ruuls et al., 2001).

In summary, these results provide the first evidence that one ADAM can compete with another ADAM for substrate processing by reducing the amount of available substrate. Moreover, we identified ADAM17 as the major PMA- and PV-stimulated sheddase for Kitl1 and Kitl2 in mEF cells. These results have implications for the functional regulation of Kitl1 and Kitl2 by ectodomain shedding, and might be particularly relevant for Kitl2 shedding, as ADAM17 is its only currently known sheddase. Since in vivo analysis of the role of ADAM17 was restricted because of the perinatal lethality of *Adam17*$^{-/-}$ mice (Peschon et al., 1998) (no defects in hematopoiesis were found in newborn mice, data not shown), future studies with conditional *Adam17*$^{-/-}$ mice as well as with mice carrying a knockin inactivating mutation of the Kitl-cleavage sites or deletion of ADAM17 activity in Kitl2 knockin mice should help to address the physiological role of ADAM17 and Kitl shedding in mouse development and in disease models.

Materials and Methods

Reagents and antibodies

All chemicals and reagents were purchased from Sigma unless indicated otherwise. Concanavalin A (ConA)-Sepharose and GammaBind-G Sepharose were purchased from Amersham Biosciences. Endo H and PNGase F were purchased from New England BioLabs. The hydroxamate-based metalloprotease inhibitor Batimastat (BB94) was kindly provided by J. David Becherer (GlaxoSmithKline, Research Triangle Park, NC). The anti-ADAM17 cytoplasmic domain antibody (Schlöndorff et al., 2000) and anti-ADAM19 antibody (Chesneau et al., 2003) have been previously described. The anti-Kitl cytoplasmic domain antibody was obtained by immunizing rabbits with a C-terminal Kitl peptide (YMLQQKEREFQEV) and used after affinity purification (Manova et al., 1992).

cDNA constructs

The cDNA encoding AP-tagged Kitl2 was constructed by inserting full-length mouse Kitl2 cDNA into the *Xho*I and *Xba*I sites of pAPtag5 vector (GenHunter Corp.) in frame, as described previously for the AP-Kitl1 construct (Chesneau *et al.,* 2003). In both cases, an AP-tag was attached to the N-terminus of the full-length wild-type protein, and a 6xHis-Myc tag was attached to the C-terminus. In order to prepare deletion mutants, site-directed mutagenesis was performed with either AP-Kitl1 or AP-Kitl2 as a template using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). All constructs were sequenced to confirm that the desired mutations or deletions had been obtained, and to rule out other mutations.

Cell culture, transfection and shedding assays

mEFs were prepared from E13.5-day embryos isolated from wild-type or ADAM-deficient mice [*Adam8*$^{-/-}$ (Kelly et al., 2005), *Adam9/12/15*$^{-/-}$ (Sahin et al., 2004), *Adam17*$^{-/-}$ (Peschon et al., 1998) and *Adam19*$^{-/-}$ (Zhou et al., 2004)] as described previously (Weskamp et al., 2002). *Adam10*$^{+/-}$ heterozygote or *Adam10*$^{-/-}$ knockout immortalized fibroblast cell lines derived from E9.5 embryos have been described previously (Hartmann et al., 2002). Both mEFs and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin. CHO cells were grown in F-12 media supplemented with 5% FCS, 2 mM L-glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin. Cells were transfected with plasmids encoding the various Kitl1 or Kitl2 constructs alone or together with mouse wild-type ADAM17, mouse wild-type ADAM19 or the catalytically inactive E>A mutant ADAM19 using Lipofectamine 2000 (Invitrogen Life Technologies). The expression of ADAM17 and ADAM19 were confirmed by western blotting as described previously (Chesneau et al., 2003; Schlöndorff et al., 2000). The AP-tagged Kitl1 or Kitl2 ectodomains released in the cell supernatants were collected and analysed in an 8% SDS-PAGE, either directly or after concentration using ConA-Sepharose lectin beads (Amersham Biosciences) followed by elution with 0.5 M α-methyl-D-mannoside in 50 mM Tris-HCl, pH 7.4 for 2 hours at 37°C (Sahin et al., 2006). The shedding of the AP-tagged ectodomain into the cell supernatant was assessed using an in-gel AP-assay as previously described for other substrates (Chesneau et al., 2003; Sahin et al., 2004; Zheng et al., 2002). Ectodomain shedding was stimulated for 1 hour with 25 ng/ml PMA or with 100 μM of the phosphatase inhibitor pervanadate (PV). Cell lysates were prepared in phosphate buffered saline (PBS), 1% Triton X-100, supplemented with a protease inhibitor cocktail (Roche). The lysates were incubated on ice for 30 minutes, and then nuclei and cell debris were removed by centrifugation at 16,000 ***g*** for 15 minutes in an Eppendorf tabletop centrifuge. 2× sample loading buffer was added prior to electrophoresis and the samples were heated to 95°C in preparation for western-blot analysis, or not heated in cases in which the AP-tag was detected after renaturation for an in-gel assay of AP activity (Sahin et al., 2006).

Pulse-chase, immunoprecipitation and enzymatic deglycosylation

Transfected COS-7 cells were used for pulse-chase experiments after overnight recovery following transfection. Cells were washed twice with PBS and then incubated for 1 hour with DMEM lacking cysteine and methionine that was supplemented with 10% dialyzed FCS. 200 μCi/ml of [^{35}S]Pro-mix (Amersham Bioscience) was then added for a 30-minute pulse-labeling period. After pulse labeling, the cells were washed twice with PBS, then chased in Opti-MEM (Invitrogen) for the indicated amounts of time, and supernatants were collected. The cells were washed twice and immediately lysed in lysis buffer [1% Triton X-100, 20 mM Tris (pH 7.5), 150 mM NaCl, 20 mM EDTA, 10% glycerol supplemented with protease inhibitor cocktail (Sigma) and 1 mM phenylmethanesulfonyl fluoride]. Cell debris was removed by centrifugation at 16,000 ***g*** for 15 minutes. The supernatants were incubated with anti-Kitl antibodies conjugated to GammaBind-G Sepharose beads (Amersham BioScience) for 2 hours. The beads were then washed once in buffer B (50 mM Tris, 500 mM NaCl, 5 mM EDTA, 0.2%

Triton X-100), three times in buffer C (50 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 5 mM EDTA) and once in buffer D (10 mM Tris, 0.1% Triton X-100). Bound material was released by incubating the beads in 5× sample buffer for 5 minutes at 95°C, separated on an 8% SDS-PAGE and visualized by autoradiography. For deglycosylation experiments, pulse-labeled cells were chased for 6 hours and then lysed and immunoprecipitated as described above. The immunoprecipitates were denatured in 60 μl of glycoprotein denaturing buffer containing 5% SDS and 10% β-mercaptoethanol by boiling for 10 minutes and divided into three tubes. For PNGase F treatment, the incubation was performed at a final concentration of 1% Nonidet P-40. The radiolabeled samples were then either mock treated or incubated with 500 units of Endo H or 500 units of PNGase F (New England BioLabs) at 37°C overnight, following the manufacturer instructions.

We thank Jacques Peschon and Roy Black (Amgen Inc., Seattle, WA) for providing *Adam17*$^{-/-}$ mice, Andy J. P. Docherty (Celltech R&D, Slough, UK) for *Adam 8*$^{-/-}$ mice, Dieter Hartmann (Leuven and Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium) and Paul Saftig (Christian-Albrechts University, Kiel, Germany) for immortalized *Adam10*$^{-/-}$ mEFs, and Yasemin Yozgat for excellent technical assistance. This study was supported by NIH-R01 GM64750 (to C.P.B.) and NIH-HL/DK55748 (to P.B.).

References

Amour, A., Knight, C., English, W., Webster, A., Slocombe, P., Knauper, V., Docherty, A., Becherer, J., Blobel, C. and Murphy, G. (2002). The enzymatic activity of ADAM8 and ADAM9 is not regulated by TIMPs. *FEBS Lett.* **524**, 154-158.

Arakawa, T., Yphantis, D. A., Lary, J. W., Narhi, L. O., Lu, H. S., Prestrelski, S. J., Clogston, C. L., Zsebo, K. M., Mendiaz, E. A., Wypych, J. et al. (1991). Glycosylated and unglycosylated recombinant-derived human stem cell factors are dimeric and have extensive regular secondary structure. *J. Biol. Chem.* **266**, 18942-18948.

Black, R., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S. et al. (1997). A metalloprotease disintegrin that releases tumour-necrosis factor-α from cells. *Nature* **385**, 729-733.

Blobel, C. P. (2005). ADAMs: key players in EGFR-signaling, development and disease. *Nat. Rev. Mol. Cell Biol.* **6**, 32-43.

Blume-Jensen, P., Claesson-Welsh, L., Siegbahn, A., Zsebo, K. M., Westermark, B. and Heldin, C. H. (1991). Activation of the human c-kit product by ligand-induced dimerization mediates circular actin reorganization and chemotaxis. *EMBO J.* **10**, 4121-4128.

Brannan, C. I., Lyman, S. D., Williams, D. E., Eisenman, J., Anderson, D. M., Cosman, D., Bedell, M. A., Jenkins, N. A. and Copeland, N. G. (1991). Steel-Dickie mutation encodes a c-kit ligand lacking transmembrane and cytoplasmic domains. *Proc. Natl. Acad. Sci. USA* **88**, 4671-4674.

Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P. and Bernstein, A. (1988). The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. *Nature* **335**, 88-89.

Cheng, H. J. and Flanagan, J. G. (1994). Transmembrane kit ligand cleavage does not require a signal in the cytoplasmic domain and occurs at a site dependent on spacing from the membrane. *Mol. Biol. Cell* **5**, 943-953.

Chesneau, V., Becherer, D., Zheng, Y., Erdjument-Bromage, H., Tempst, P. and Blobel, C. P. (2003). Catalytic properties of ADAM19. *J. Biol. Chem.* **278**, 22331-22340.

Copeland, N. G., Gilbert, D. J., Cho, B. C., Donovan, P. J., Jenkins, N. A., Cosman, D., Anderson, D., Lyman, S. D. and Williams, D. E. (1990). Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. *Cell* **63**, 175-183.

Cruz, A. C., Frank, B. T., Edwards, S. T., Dazin, P. F., Peschon, J. J. and Fang, K. C. (2004). Tumor necrosis factor-alpha-converting enzyme controls surface expression of c-Kit and survival of embryonic stem cell-derived mast cells. *J. Biol. Chem.* **279**, 5612-5620.

Fambrough, D., Pan, D., Rubin, G. M. and Goodman, C. S. (1996). The cell surface metalloprotease/disintegrin kuzbanian is required for axonal extension in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **93**, 13233-13238.

Flanagan, J. G., Chan, D. C. and Leder, P. (1991). Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sld mutant. *Cell* **64**, 1025-1035.

Geissler, E. N., Ryan, M. A. and Housman, D. E. (1988). The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. *Cell* **55**, 185-192.

Hartmann, D., de Strooper, B., Serneels, L., Craessaerts, K., Herreman, A., Annaert, W., Umans, L., Lubke, T., Lena Illert, A., von Figura, K. et al. (2002). The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. *Hum. Mol. Genet.* **11**, 2615-2624.

Hattori, M., Osterfield, M. and Flanagan, J. G. (2000). Regulated cleavage of a contact-mediated axon repellent. *Science* **289**, 1360-1365.

Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N. R., Crystal, R. G., Besmer, P., Lyden, D., Moore, M. A. et al. (2002). Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* **109**, 625-637.

Hooper, N. M., Karran, E. H. and Turner, A. J. (1997). Membrane protein secretases. *Biochem. J.* **321**, 265-279.

Horiuchi, K., Weskamp, G., Lum, L., Hammes, H. P., Cai, H., Brodie, T. A., Ludwig, T., Chiusaroli, R., Baron, R., Preissner, K. T. et al. (2003). Potential role for ADAM15 in pathological neovascularization in mice. *Mol. Cell. Biol.* **23**, 5614-5624.

Horiuchi, K., Zhou, H. M., Kelly, K., Manova, K. and Blobel, C. P. (2005). Evaluation of the contributions of ADAMs 9, 12, 15, 17, and 19 to heart development and ectodomain shedding of neuregulins beta1 and beta2. *Dev. Biol.* **283**, 459-471.

Horiuchi, K., Le Gall, S., Schulte, M., Yamaguchi, T., Reiss, K., Murphy, G., Toyama, Y., Hartmann, D., Saftig, P. and Blobel, C. P. (2007). Substrate selectivity of EGF-receptor ligand sheddases and their regulation by phorbol esters and calcium influx. *Mol. Biol. Cell.* **18**, 176-188.

Huang, E., Nocka, K., Beier, D. R., Chu, T. Y., Buck, J., Lahm, H. W., Wellner, D., Leder, P. and Besmer, P. (1990). The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. *Cell* **63**, 225-233.

Huang, E. J., Nocka, K. H., Buck, J. and Besmer, P. (1992). Differential expression and processing of two cell associated forms of the *kit*-ligand: KL-1 and KL-2. *Mol. Biol. Cell* **3**, 349-362.

Jackson, L. F., Qiu, T. H., Sunnarborg, S. W., Chang, A., Zhang, C., Patterson, C. and Lee, D. C. (2003). Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. *EMBO J.* **22**, 2704-2716.

Jiang, X., Gurel, O., Mendiaz, E. A., Stearns, G. W., Clogston, C. L., Lu, H. S., Osslund, T. D., Syed, R. S., Langley, K. E. and Hendrickson, W. A. (2000). Structure of the active core of human stem cell factor and analysis of binding to its receptor kit. *EMBO J.* **19**, 3192-3203.

Kawaguchi, N., Xu, X., Tajima, R., Kronqvist, P., Sundberg, C., Loechel, F., Albrechtsen, R. and Wewer, U. M. (2002). ADAM 12 protease induces adipogenesis in transgenic mice. *Am. J. Pathol.* **160**, 1895-1903.

Kawaguchi, N., Sundberg, C., Kveiborg, M., Moghadaszadeh, B., Asmar, M., Dietrich, N., Thodeti, C. K., Nielsen, F. C., Moller, P., Mercurio, A. M. et al. (2003). ADAM12 induces actin cytoskeleton and extracellular matrix reorganization during early adipocyte differentiation by regulating beta1 integrin function. *J. Cell Sci.* **116**, 3893-3904.

Kelly, K., Hutchinson, G., Klewe-Nebenius, D., Smith, A., Bartsch, J. W., Horiuchi, K., Manova, K., Docherty, A. J. and Blobel, C. P. (2005). Metalloprotese-disintegrin ADAM8: Expression analysis and targeted deletion in mice. *Dev. Dyn.* **232**, 221-231.

Kurohara, K., Komatsu, K., Kurisaki, T., Masuda, A., Irie, N., Asano, M., Sudo, K., Nabeshima, Y., Iwakura, Y. and Sehara-Fujisawa, A. (2004). Essential roles of Meltrin beta (ADAM19) in heart development. *Dev. Biol.* **267**, 14-28.

Kveiborg, M., Frohlich, C., Albrechtsen, R., Tischler, V., Dietrich, N., Holck, P., Kronqvist, P., Rank, F., Mercurio, A. M. and Wewer, U. M. (2005). A role for ADAM12 in breast tumor progression and stromal cell apoptosis. *Cancer Res.* **65**, 4754-4761.

Langley, K. E., Wypych, J., Mendiaz, E. A., Clogston, C. L., Parker, V. P., Farrar, D. H., Brothers, M. O., Satygal, V. N., Leslie, I., Birkett, N. C. et al. (1992). Purification and characterization of soluble forms of human and rat stem cell factor recombinantly expressed by Escherichia coli and by Chinese hamster ovary cells. *Arch. Biochem. Biophys.* **295**, 21-28.

Langley, K. E., Mendiaz, E. A., Liu, N., Narhi, L. O., Zeni, L., Parseghian, C. M., Clogston, C. L., Leslie, I., Pope, J. A., Lu, H. S. et al. (1994). Properties of variant forms of human stem cell factor recombinantly expressed in Escherichia coli. *Arch. Biochem. Biophys.* **311**, 55-61.

Lev, S., Yarden, Y. and Givol, D. (1992). Dimerization and activation of the kit receptor by monovalent and bivalent binding of the stem cell factor. *J. Biol. Chem.* **267**, 15970-15977.

Longley, B. J., Tyrrell, L., Ma, Y., Williams, D. A., Halaban, R., Langley, K., Lu, H. S. and Schechter, N. M. (1997). Chymase cleavage of stem cell factor yields a bioactive, soluble product. *Proc. Natl. Acad. Sci. USA* **94**, 9017-9021.

Majumdar, M. K., Feng, L., Medlock, E., Toksoz, D. and Williams, D. A. (1994). Identification and mutation of primary and secondary proteolytic cleavage sites in murine stem cell factor cDNA yields biologically active, cell-associated protein. *J. Biol. Chem.* **269**, 1237-1242.

Manova, K., Bachvarova, R. F., Huang, E. J., Sanchez, S., Pronovost, S. M., Velazquez, E., McGuire, B. and Besmer, P. (1992). c-kit receptor and ligand expression in postnatal development of the mouse cerebellum suggests a function for c-kit in inhibitory interneurons. *J. Neurosci.* **12**, 4663-4676.

Maskos, K., Fernandez-Catalan, C., Huber, R., Bourenkov, G. P., Bartunik, H., Ellestad, G. A., Reddy, P., Wolfson, M. F., Rauch, C. T., Castner, B. J. et al. (1998). Crystal structure of the catalytic domain of human tumor necrosis factor-alpha-converting enzyme. *Proc. Natl. Acad. Sci. USA* **95**, 3408-3412.

Merlos-Suarez, A., Ruiz-Paz, S., Baselga, J. and Arribas, J. (2001). Metalloprotease-dependent protransforming growth factor-alpha ectodomain shedding in the absence of tumor necrosis factor-alpha-converting enzyme. *J. Biol. Chem.* **276**, 48510-48517.

Mohan, M., Seaton, T., Mitchell, J., Howe, A., Blackburn, K., Burkhart, W., Moyer, M., Patel, I., Becherer, J., Moss, M. et al. (2002). The tumor necrosis factor alpha converting enzyme (TACE): a unique metalloproteinase with highly defined substrate selectivity. *Biochemistry* **41**, 9462-9469.

Moss, M. L., Jin, S.-L. C., Milla, M. E., Burkhart, W., Cartner, H. L., Chen, W.-J., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R. et al. (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. *Nature* **385**, 733-736.

Nocka, K., Buck, J., Levi, E. and Besmer, P. (1990). Candidate ligand for the c-kit

transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. *EMBO J.* **9**, 3287-3294.

Pandiella, A., Bosenberg, M., Huang, E. J., Besmer, P. and Massague, J. (1992). Cleavage of membrane-anchored growth factors involves distinct protease activities regulated through common mechanisms. *J. Biol. Chem.* **267**, 24028-24033.

Peduto, L., Reuter, V. E., Shaffer, D. R., Scher, H. I. and Blobel, C. P. (2005). Critical function for ADAM9 in mouse prostate cancer. *Cancer Res.* **65**, 9312-9319.

Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russel, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N. et al. (1998). An essential role for ectodomain shedding in mammalian development. *Science* **282**, 1281-1284.

Primakoff, P. and Myles, D. G. (2000). The ADAM gene family: surface proteins with an adhesion and protease activity packed into a single molecule. *Trends Genet.* **16**, 83-87.

Roghani, M., Becherer, J. D., Moss, M. L., Atherton, R. E., Erdjument-Bromage, H., Arribas, J., Blackburn, R. K., Weskamp, G., Tempst, P. and Blobel, C. P. (1999). Metalloprotease-disintegrin MDC9: intracellular maturation and catalytic activity. *J. Biol. Chem.* **274**, 3531-3540.

Russell, E. S. (1979). Hereditary anemias of the mouse: a review for geneticists. *Adv. Genet.* **20**, 357-459.

Ruuls, S. R., Hoek, R. M., Ngo, V. N., McNeil, T., Lucian, L. A., Janatpour, M. J., Korner, H., Scheerens, H., Hessel, E. M., Cyster, J. G. et al. (2001). Membrane-bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. *Immunity* **15**, 533-543.

Sahin, U., Weskamp, G., Zhou, H. M., Higashiyama, S., Peschon, J. J., Hartmann, D., Saftig, P. and Blobel, C. P. (2004). Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR-ligands. *J. Cell Biol.* **164**, 769-779.

Sahin, U., Weskamp, G., Zheng, Y., Chesneau, V., Horiuchi, K. and Blobel, C. P. (2006). A sensitive method to monitor ectodomain shedding of ligands of the epidermal growth factor receptor. In *Epidermal Growth Factor: Methods and Protocols*. Vol. 327 (ed. P. J. Bertics and T. B. Patel), pp. 99-113. Totowa, NJ: Humana Press.

Schlomann, U., Wildeboer, D., Webster, A., Antropova, O., Zeuschner, D., Knight, C. G., Docherty, A. J., Lambert, M., Skelton, L., Jockusch, H. et al. (2002). The metalloprotease disintegrin ADAM8. Processing by autocatalysis is required for proteolytic activity and cell adhesion. *J. Biol. Chem.* **277**, 48210-48219.

Schlöndorff, J. and Blobel, C. P. (1999). Metalloprotease-disintegrins: modular proteins capable of promoting cell-cell interactions and triggering signals by protein ectodomain shedding. *J. Cell. Sci.* **112**, 3603-3617.

Schlöndorff, J., Becherer, J. D. and Blobel, C. P. (2000). Intracellular maturation and localization of the tumour necrosis factor alpha convertase (TACE). *Biochem. J.* **347**, 131-138.

Seals, D. F. and Courtneidge, S. A. (2003). The ADAMs family of metalloproteases: multidomain proteins with multiple functions. *Genes Dev.* **17**, 7-30.

Silvers, W. K. (1979). *The Coat Colors of Mice: A Model for Gene Action and Interaction*. New York: Springer-Verlag.

Sternlicht, M. D., Sunnarborg, S. W., Kouros-Mehr, H., Yu, Y., Lee, D. C. and Werb, Z. (2005). Mammary ductal morphogenesis requires paracrine activation of stromal EGFR via ADAM17-dependent shedding of epithelial amphiregulin. *Development* **132**, 3923-3933.

Stocker, W., Grams, F., Baumann, U., Reinemer, P., Gomis-Ruth, F. X., McKay, D. B. and Bode, W. (1995). The metzincins–topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. *Protein Sci.* **4**, 823-840.

Sunnarborg, S. W., Hinkle, C. L., Stevenson, M., Russell, W. E., Raska, C. S., Peschon, J. J., Castner, B. J., Gerhart, M. J., Paxton, R. J., Black, R. A. et al. (2002). Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. *J. Biol. Chem.* **277**, 12838-12845.

Tajima, Y., Moore, M. A., Soares, V., Ono, M., Kissel, H. and Besmer, P. (1998). Consequences of exclusive expression in vivo of Kit-ligand lacking the major proteolytic cleavage site. *Proc. Natl. Acad. Sci. USA* **95**, 11903-11908.

Van Eerdewegh, P., Little, R. D., Dupuis, J., Del Mastro, R. G., Falls, K., Simon, J., Torrey, D., Pandit, S., McKenny, J., Braunschweiger, K. et al. (2002). Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature* **418**, 426-430.

Weskamp, G., Cai, H., Brodie, T. A., Higashyama, S., Manova, K., Ludwig, T. and Blobel, C. P. (2002). Mice lacking the metalloprotease-disintegrin MDC9 (ADAM9) have no evident major abnormalities during development or adult life. *Mol. Cell. Biol.* **22**, 1537-1544.

White, J. M. (2003). ADAMs: modulators of cell-cell and cell-matrix interactions. *Curr. Opin. Cell Biol.* **15**, 598-606.

Yamazaki, S., Iwamoto, R., Saeki, K., Asakura, M., Takashima, S., Yamazaki, A., Kimura, R., Mizushima, H., Moribe, H., Higashiyama, S. et al. (2003). Mice with defects in HB-EGF ectodomain shedding show severe developmental abnormalities. *J. Cell Biol.* **163**, 469-475.

Zhang, Z., Zhang, R., Joachimiak, A., Schlessinger, J. and Kong, X. P. (2000). Crystal structure of human stem cell factor: implication for stem cell factor receptor dimerization and activation. *Proc. Natl. Acad. Sci. USA* **97**, 7732-7737.

Zheng, Y., Schlöndorff, J. and Blobel, C. P. (2002). Evidence for regulation of the tumor necrosis factor alpha-convertase (TACE) by protein-tyrosine phosphatase PTPH1. *J. Biol. Chem.* **277**, 42463-42470.

Zheng, Y., Saftig, P., Hartmann, D. and Blobel, C. (2004). Evaluation of the contribution of different ADAMs to TNFα shedding and of the function of the TNFα ectodomain in ensuring selective stimulated shedding by the TNFα convertase (TACE/ADAM17). *J. Biol. Chem.* **279**, 42898-42906.

Zhou, H. M., Weskamp, G., Chesneau, V., Sahin, U., Vortkamp, A., Horiuchi, K., Chiusaroli, R., Hahn, R., Wilkes, D., Fisher, P. et al. (2004). Essential role for ADAM19 in cardiovascular morphogenesis. *Mol. Cell. Biol.* **24**, 96-104.

Zou, J., Zhu, F., Liu, J., Wang, W., Zhang, R., Garlisi, C. G., Liu, Y. H., Wang, S., Shah, H., Wan, Y. et al. (2004). Catalytic activity of human ADAM33. *J. Biol. Chem.* **279**, 9818-9830.

Zsebo, K. M., Williams, D. A., Geissler, E. N., Broudy, V. C., Martin, F. H., Atkins, H. L., Hsu, R. Y., Birkett, N. C., Okino, K. H., Murdock, D. C. et al. (1990). Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* **63**, 213-224.