

Proteolytic regulation and functional repertoire of soluble ADAM17

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Table of Contents

Abstract.....	7
Zusammenfassung	8
1. Introduction.....	10
1.1 Proteolysis and ectodomain shedding	10
1.1.1 Metalloproteases	10
1.1.2 ADAMs	11
1.2 ADAM17	13
1.2.1 ADAM17 in EGFR signaling mediated pathologies.....	15
1.2.2 Regulation of ADAM17	17
1.2.2.1 Regulation by iRhoms	18
1.2.2.2 Regulation by inhibition.....	22
1.2.2.3 Regulation by glycosylation	23
1.3 ADAM8	24
1.3.1 Proteolytic and non-proteolytic functions of ADAM8	25
1.3.2 Regulation of ADAM8	26
1.3.3 Proteolytic generation of sADAM17 via ADAM8	26
1.4 ADAM17 and ADAM8 in breast cancer	28
1.5 Aim of the study	31
2. Material and Methods.....	32
2.1 Chemicals.....	32
2.2 Buffers and solutions	32
2.3 Selection antibiotics and inhibitors	34
2.4 Heat shock transformation and plasmid scale-up	34
2.5 Cloning	35
2.5.1 Cloning of membrane-bound mADAM17 with HA-tag after pro-domain.....	35
2.5.2 Cloning of ectodomain of murine ADAM17	37
2.6 Cell culture	39
2.7 Transient transfection	39
2.8 Retroviral transduction	41

2.9 Cell lysis and TCA precipitation	41
2.10 SDS-PAGE and Western blot	42
2.11 Immunofluorescence	44
2.12 Glycoprotein enrichment and deglycosylation	45
2.13 AP-activity assay	45
2.14 Cell surface biotinylation	46
2.15 Fluorogenic peptide-based activity assay	46
2.16 N-terminomics for new substrate identification	47
2.16.1 Sample preparation for TAILS analysis	47
2.16.2 TAILS	49
2.16.3 LC-MS/MS analysis	49
2.16.4 Data analysis and normalization	50
2.17 Statistical analysis and illustrations	51
3. Results	52
3.1 ADAM8 mediated shedding of ADAM17 in breast cancer cells	52
3.1.1 Interaction of ADAM17 and ADAM8 on the cell surface	52
3.1.2 Colocalization of ADAM17 and ADAM8 in the secretory pathway	55
3.1.3 Shedding of ADAM17 in the presence of protease or trafficking inhibitors	57
3.1.4 Proteolytically released soluble ADAM17-mediated peptide processing	60
3.2 Shedding of ADAM17 in the presence or absence of iRhom1/2	62
3.2.1 Generation of stably transfected mEF cells	62
3.2.2 Generation and characterization of murine ADAM17 with HA-tag after pro-domain	64
3.2.3 Impact of iRhom2 and its phosphorylation on the shedding of ADAM17	67
3.3 Shedding functions of soluble ADAM17	69
3.3.1 Characterization of soluble ADAM17	69
3.3.2 EGFR ligand shedding by soluble ADAM17	75
3.3.3 EGFR ligand shedding by proteolytically released soluble ADAM17	78
3.3.4 EGFR ligand shedding in the absence of iRhom1/2	81

3.4 Identification of putative new substrates of ADAM8 and ADAM17 in breast cancer cells	85
3.5 Identification of new substrates of sADAM17	89
4. Discussion	93
4.1 Shedding of ADAM17 occurs majorly in the secretory pathway	94
4.2 iRhom1/2 do not participate in the shedding and maturation of sADAM17	95
4.3 Expression of catalytically active, soluble ADAM17	98
4.4 sADAM17 is capable of shedding EGFR ligands in a substrate-dependent manner	99
4.5 sADAM17 sheds AREG and HB-EGF independent of iRhom1/2	99
4.6 Potential physiological roles of sADAM17 in triple-negative breast cancer	101
4.7 Summary and Outlook	104
5. References	106
6. Supplementary Information	126
Declaration of Authorship	146
Acknowledgments	147
Appendix	148
I. Abbreviations	148

List of Figures

Figure 1: General structure of ADAM proteases.....	11
Figure 2: ADAM17 in physiological and pathological processes	15
Figure 3: Schematic representation of ADAM17 function in EGFR signaling	16
Figure 4: iRhoms in maturation and trafficking of ADAM17.....	20
Figure 5: Regulation of ADAM17 activity by iRhom2	22
Figure 6: Part-time sheddase model of ADAM17.....	28
Figure 7: Scheme for sample preparation for N-terminomics analysis	48
Figure 8: Cell surface colocalization of ADAM8 and ADAM17 visualized by immunofluorescence microscopy	53
Figure 9: Cell surface protein biotinylation in MDA-MB-231 cells.....	54
Figure 10: Colocalization of ADAM8 and ADAM17 in the secretory pathway.....	56
Figure 11: Predicted outcome for the shedding of ADAM17 in the presence of inhibitors	57
Figure 12: Shedding of endogenous and overexpressed human ADAM17 in the presence of inhibitors.....	60
Figure 13: Proteolytic activity of shed hADAM17	62
Figure 14: Stable transfection of iRhom1/2 ^{-/-} mEF cells.....	63
Figure 15: Biochemical properties of murine ADAM17 with HA-tag after pro-domain.....	65
Figure 16: Biotinylation of cell surface ADAM17 in iRhom1/2 ^{-/-} mEFs.....	66
Figure 17: iRhom2 and iRhom2 phosphorylation independent shedding of ADAM17	68
Figure 18: Biochemical characterization of soluble ADAM17.....	71
Figure 19: Cell surface biotinylation of mADAM17 variants	72
Figure 20: Soluble ADAM17 is a complex/fully glycosylated protease	74
Figure 21: sADAM17 is capable of shedding different EGFR ligands in <i>cis</i>	77
Figure 22: Alkaline-phosphatase activity by sEGFR ligands released in <i>cis</i>	77
Figure 23: sADAM17 is capable of shedding different EGFR ligands in <i>trans</i>	80
Figure 24: Comparative alkaline-phosphatase activity by sEGFR ligands released in <i>cis</i> and <i>trans</i>	80
Figure 25: sADAM17-mediated AREG-AP shedding in the presence or absence of iRhom1/2	82
Figure 26: sADAM17-mediated HB-EGF-AP shedding in the presence or absence of iRhom1/2	84
Figure 27: N-terminomics TAILS analysis scheme	86
Figure 28: Novel endogenous substrate of ADAM8 and ADAM17	89
Figure 29: Novel endogenous substrate of ADAM8 and ADAM17	91
Figure 30: A predicted pathway for iRhom independent shedding of ADAM17	97
Figure 31: Potential Physiological relevance of sADAM17.....	105
Supplementary Figure 1: GO annotation and Reactome analysis via Metascape	145

List of Tables

Table 1: Overview of buffers and solutions used for the experiments	32
Table 2: Overview of antibiotics and inhibitors	34
Table 3: List of cloning primers for mADAM17 PD_HA.....	35
Table 4: Components of the PCR reaction mix	36
Table 5: PCR program scheme	36
Table 6: Components of fusion PCR for mADAM17 PD_HA	37
Table 7: Fusion PCR program scheme for mADAM17 PD_HA	37
Table 8: List of cloning primers for HA_sADAM17_flag	38
Table 9: Components of fusion PCR for sHA_mADAM17_flag	38
Table 10: Overview of plasmids used for transient transfection.....	40
Table 11: Resolving and stacking gel recipe for SDS-PAGE analysis.....	43
Table 12: List of primary antibodies used in western blot analysis	43
Table 13: List of secondary antibodies used in western blot analysis	44
Table 14: List of primary antibodies used in immunofluorescence microscopy	44
Table 15: List of secondary antibodies used in immunofluorescence microscopy	45
 Supplementary table 1: GO functions, Reactome, Kegg Pathway and Biological process analysis via Metascpae for top 30 proteins upregulated in WT MDA-MB-231 cells against hA8 KO, hA8 E>Q cells	 126
Supplementary table 2: Metascape analysis of top 30 upregulated proteins in hA8 KO MDA- MB-231 cells with conditioned media (hA8 KO+CM) against hA8 KO and hA8 KO+CM+GW cells.....	135

Abstract

A disintegrin and metalloprotease 17 (ADAM17) is a membrane-bound zinc metalloprotease. It is one of the major sheddases that plays key role in cellular events like tumorigenesis, inflammation, and cell homeostasis by cleaving off the ectodomains of several membrane-bound proteins like Tumor necrosis factor- α , Interleukin-6 receptor and Epidermal growth factor receptor (EGFR) ligands. Therefore, the regulation of the proteolytic activity of ADAM17 is an essential process. Notably, the inactivated rhomboid proteins (iRhoms)1 and 2 were shown to be indispensable for the trafficking and maturation of ADAM17. Additionally, iRhom1 and iRhom2 are also involved in the mechanism of substrate recruitment and specificity on the cell surface for membrane-bound ADAM17-mediated shedding. Intriguingly, the proteolytic release of the ADAM17 ectodomain (sADAM17) itself was only recently addressed, where ADAM8 was identified as a sheddase of ADAM17.

In this thesis, the proteolytic release of sADAM17 was analyzed via *in vitro* experiments for endogenous and overexpressed, human, and murine ADAM17. Furthermore, the role of iRhom1/2 was explored pertaining to the shedding of ADAM17 and its subsequent maturation and activation. The shedding of ADAM17 commences in the secretory pathway and is independent of iRhom1/2. Contrasting to membrane-bound ADAM17, sADAM17 gets matured and activated independent of iRhoms, attaining all the necessary post-translational modifications.

So far, the initial studies with the recombinant soluble ADAM17 provided some insights into its additional functions within the protease-web as a soluble enzyme. Therefore, it is important to understand the mechanism and regulation of the proteolytic release of soluble ADAM17 and its consequent functions. Thus, the second part of the thesis dealt with the functional aspects of sADAM17. Interestingly, a persisting shedding capacity of sADAM17 was detected *in vitro* against EGFR ligands in a substrate-dependent manner as compared to its membrane-bound counterpart. Of note, the shedding of amphiregulin and heparin binding-epidermal growth factor by sADAM17 appeared to be independent of iRhom1/2, unlike shedding via membrane-bound ADAM17. Finally, the application of N-terminomics in triple-negative breast cancer cells revealed further substrates and potential roles of sADAM17 in extracellular matrix remodeling and consequent invasion and metastasis. These findings altogether indicate a novel regulatory mechanism of sADAM17 demonstrating its ability to process substrate in close proximity to the plasma membrane and beyond its cellular vicinity. These insights enhance the understanding of the intricate molecular mechanisms underlying sADAM17-mediated cellular functions and disease pathways while exhibiting part-time sheddase properties.

Zusammenfassung

A disintegrin and metalloprotease 17 (ADAM17) ist eine membrangebundene Zink-abhängige Metalloprotease. ADAM17 ist von zentraler funktioneller Bedeutung in Prozessen wie der Entstehung von Tumoren, Entzündung und Zellhomöostase, indem es die Ektodomänen membrangebundener Proteine wie Tumor necrosis factor- α , Interleukin-6-Rezeptor sowie Liganden des Epidermal growth factor receptors (EGFR) proteolytisch abspaltet. Die Regulation der proteolytischen Aktivität von ADAM17 zu verstehen ist daher von großem Interesse. In diesem Kontext wurden die inaktivierten Rhomboid-Proteine (iRhom)1 und 2 als unverzichtbar für den Transport und die Reifung von ADAM17 identifiziert. Darüber hinaus sind iRhom1 und iRhom2 auch an der Rekrutierung von Substraten beteiligt und beeinflussen somit die Spezifität von ADAM17 auf der Zelloberfläche. Einen weiteren erst kürzlich beschriebenen regulatorischen Mechanismus, stellt die proteolytische Abspaltung der ADAM17-Ektodomäne (sADAM17) durch ADAM8 dar.

In der vorliegenden Arbeit wurde *in vitro* die proteolytische Abspaltung von sADAM17 ausgehend von endogenem sowie überexprimiertem ADAM17 humanen und murinen Ursprungs analysiert. Außerdem wurde die Rolle von iRhom1/2 in Bezug auf die Freisetzung von sADAM17 sowie dessen nachfolgende Reifung und Aktivierung untersucht. Die Bildung von sADAM17 erfolgt auf dem sekretorischen Weg und ist unabhängig von iRhom1/2. Im Gegensatz zu membrangebundenem ADAM17 wird sADAM17 unabhängig von iRhoms gebildet und aktiviert, wobei es alle notwendigen posttranslationalen Modifikationen erhält.

Bisherige Studien mit rekombinantem, löslichem ADAM17 ermöglichten Einblicke in zusätzliche Funktionen als lösliches Enzym innerhalb des Protease-Netzwerks. Die Erkenntnisse aus diesen Untersuchungen eröffneten ein bisher unbekanntes funktionelles Spektrum von ADAM17, welches die Notwendigkeit bedingt den Mechanismus und die Regulation der proteolytischen Abspaltung von sADAM17 besser zu verstehen. Im zweiten Teil dieser Arbeit wurden daher die funktionalen Aspekte von sADAM17 untersucht. Interessanterweise zeigten *in vitro* Untersuchungen, dass auch sADAM17 in der Lage war, einige EGFR-Liganden proteolytisch von der Zelloberfläche freizusetzen. Bemerkenswert ist, dass die Abspaltung von Amphiregulin und Heparin-binding-EGF-like growth factor durch sADAM17 unabhängig von iRhom1/2 war, im Gegensatz zur Abspaltung durch membrangebundenes ADAM17. Mittels N-Terminomics Analysen an TNBC-Zellen wurden weitere Substrate identifiziert, die auf potenzielle Rollen von sADAM17 bei der Umgestaltung der extrazellulären Matrix und somit der Invasion und Metastasierung von Krebszellen hinweisen. Die Erkenntnisse der vorliegenden Arbeit deuten insgesamt auf einen neuen Regulationsmechanismus von sADAM17 hin, welcher sowohl die Prozessierung von Substraten in der Nähe der Plasmamembran als auch außerhalb des Zellumfelds ermöglicht.

Diese Einsichten vertiefen somit das Verständnis der komplexen molekularen Mechanismen, die sADAM17-vermittelten zellulären Funktionen und potenziell assoziierten Krankheitsmechanismen zugrunde liegen.

1. Introduction

1.1 Proteolysis and ectodomain shedding

The hydrolysis of peptide bonds of a protein to generate smaller polypeptides is known as proteolysis. Limited proteolysis is an irreversible post-translational modification (PTM) of the target protein. This highly specific PTM is a well-controlled process for the regulation of numerous physiological and pathophysiological cellular processes such as development, tumorigenesis, neurodegeneration, apoptosis, proliferation, etc. (López-Otín & Overall, 2002). The proteolytic cleavage of membrane proteins, like cytokines, adhesion molecules, and growth factors, involved in these aforementioned cellular events within the transmembrane (TM) domain or close to the membrane resulting in the release of the bulk or even the entire extracellular domain (ectodomain) is called shedding (Lambrecht et al., 2018; Lemberg, 2011; Lichtenthaler & Steiner, 2007; McCarthy et al., 2017). Proteases that mediate shedding are therefore also referred to as sheddases and can either be membrane-bound or soluble (Lichtenthaler et al., 2018). Shedding can take place at the plasma membrane and in all cellular organelles of the secretory and endocytic pathways (Lichtenthaler et al., 2018). The proteases involved in ectodomain shedding are defined as sheddases (Peschon et al., 1998). Some shedding proteases such as a disintegrin and metalloprotease (ADAM) and β -site amyloid precursor protein cleaving enzyme (BACE) are termed “full-time sheddases” based on their primary function to release the ectodomains of their membrane-bound substrates. On the other hand, the term “part-time sheddases” was coined to define the proteases that typically show non-shedding functions by cleaving soluble substrates but can act on membrane-bound substrates by cleaving in the juxtamembrane domain (Lichtenthaler et al., 2018). One such example of “part-time sheddases” is a soluble matrix metalloprotease, MMP9 that sheds neuroglycin 1 in the nervous system (Peixoto et al., 2012). Another instance is soluble meprin β , which after its release from the cell surface can reach to distant substrate pool as compared to membrane-bound meprin β (Wichert et al., 2017).

1.1.1 Metalloproteases

The proteolytic processing is carried out by proteases, which are classified according to the key elements of the catalytic mechanism into aspartic, cysteine, metallo, serine, and threonine proteases (Kappelhoff et al., 2017; López-Otín & Matrisian, 2007; Rawlings & Barrett, 1993). Proteases containing metal ions in the active site are known as metalloproteases. Most metalloproteases contain a zinc metal ion complexed in the active site cleft of the enzyme and are called zinc-metalloproteases (Rawlings & Barrett, 1993). Furthermore, the metalloprotease class is divided into sub-class: metzincins; a superfamily that is characterized by a long zinc-

binding consensus sequence: HEXXHXXGXXH followed by a methionine turn (Met-turn). The shared characteristic consensus motif HEBXHBGBXH carries three histidine (H) residues that bind to the zinc ion, a catalytically active glutamate (E) residue, few hydrophobic bulky residues (B), a glycine (G) residue and X as any amino acid residue (Bode et al., 1993; Hooper, 1994; Stöcker et al., 1995). The metalloproteases grouped into this common family are; a disintegrin and metalloproteases (ADAMs), ADAMs with thrombospondin motifs (ADAMTS), matrix metalloproteases (MMPs), astacins, and other 9 metalloproteases (Bode et al., 1993; Cerda-Costa & Gomis-Ruth, 2014; Gomis-Rüth, 2003; Hooper, 1994). In the following, ADAM proteases are further described.

1.1.2 ADAMs

ADAMs were first discovered to play a functional role in sperm-egg fusion in the guinea pig (Blobel et al., 1992). Out of the 22 identified human ADAMs, 12 are active human sheddases (ADAM8, 9, 10, 12, 15, 17, 19, 20, 21, 28, 30, and 33) that share the classic consensus sequence for binding the zinc metal ion in the active site (Bode et al., 1993; Mullooly et al., 2016; Murphy, 2008 ; Weber & Saftig, 2012). Prototypically, ADAMs are multidomain type-1 membrane proteins (Figure 1): they typically consist of an N-terminal signal peptide followed by a pro-domain (PD) attached to the catalytic metalloprotease domain (CD), a disintegrin domain (DIS), a cysteine-rich domain followed by an EGF-like domain consisting of 1-4 EGF repeats, a transmembrane domain (TMD), and finally a cytoplasmic C-terminal domain (CTD). Exceptionally, in ADAM10 and ADAM17, the cysteine-rich domain and EGF-like domain are replaced by a membrane-proximal domain (MPD) followed by a short stalk region (Düsterhöft et al., 2013; Lambrecht et al., 2018).

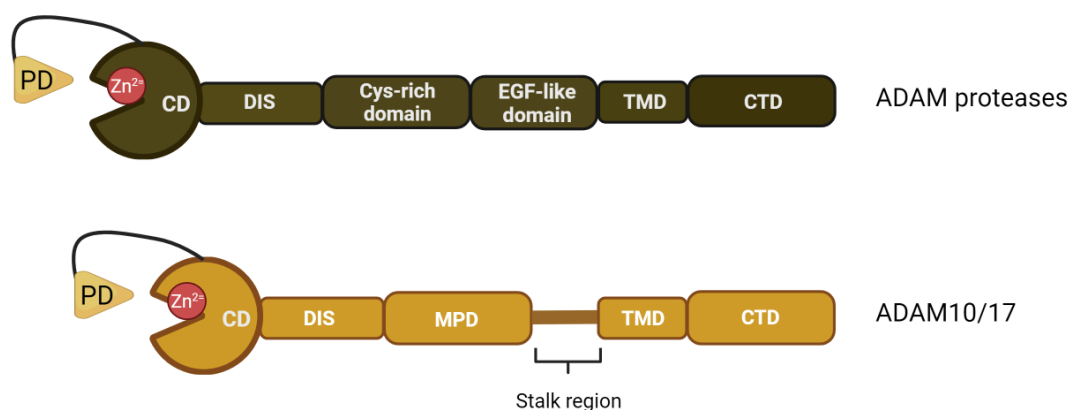


Figure 1: General structure of ADAM proteases

ADAMs are multidomain proteins consisting of a pro-domain (PD), catalytic domain carrying zinc ion (CD), Disintegrin domain (DIS), a cysteine-rich domain, an EGF-like domain consisting of 1-4 EGF-like repeats a transmembrane domain (TMD) and a cytoplasmic domain (CTD). In the case of ADAM10/17, the disintegrin domain is followed by a membrane-proximal domain (MPD) and a stalk region. Modified from (Düsterhöft et al., 2013; Lambrecht et al., 2018; Seals & Courtneidge, 2003).

The signal peptide directs the protein into the secretory pathway, and the pro-domain keeps the protease in an inactive state until it is removed by proprotein convertases (PCs), such as furin, by cleaving at the RX(K/R)R motif during the transit through the Golgi network (Howard et al., 2000; Nakayama, 1997; Wong et al., 2015). Of note, a few ADAMs such as ADAM8 and ADAM28, which lack this consensus sequence, autocatalytically remove their pro-domain (Howard et al., 2000; Schlomann et al., 2002). Additionally, the pro-domain is also reported to control proper protein folding and act as a chaperone to assist in secretion (Leonard et al., 2005; Seals & Courtneidge, 2003). The disintegrin-like domain can interact with adhesion molecules of the integrin family and participate in cell migration (Arribas et al., 2006; Huang et al., 2005). The cysteine-rich domain reportedly binds to cell surface syndecans to promote cell spreading (Iba et al., 2000). CTD is known as a region of variable length and sequence. It interacts with intracellular proteins to regulate protein functions and localization via its phosphorylation sites binding to Src-homology-3 (SH3) domain-containing proteins (Blobel, 2005; Lambrecht et al., 2018; Seals & Courtneidge, 2003). Proteolytically active ADAMs have been extensively studied as important sheddases, and the released soluble ectodomains of their substrates are involved in endo- and paracrine-signaling (Blobel, 2005). Due to their regulatory function in various physiological processes such as muscle development, fertilization, tissue regeneration and cell fate determination, as well as in pathologies such as inflammation and cancer, ADAMs are also referred to as important molecular scissors. (Arai et al., 2023; Lichtenthaler et al., 2018; Rahn & Becker-Pauly, 2022). Complete deletion or targeted disruption of functionally active ADAMs like ADAM10 (Hartmann et al., 2002), ADAM17 (Peschon et al., 1998), ADAM19 (Zhou et al., 2004), and ADAM22 (Sagane et al., 2005) resulted in severe developmental defects or even early death in mice. In contrast, mice lacking ADAM8 (Kelly et al., 2005), ADAM9 (Izumi et al., 1998), or simultaneously ADAM9/12/15 (Horiuchi et al., 2005) were viable, fertile, and normal development was not impaired.

The most well-known transmembrane substrates are tumor necrosis factor- α (TNF- α) and Notch (Amour et al., 2002; Black et al., 1997; Hartmann et al., 2002), ligands of epidermal growth factor receptor (EGFR) like transforming growth factor- α (TGF- α), amphiregulin (AREG), betacellulin (BTC), etc., (Sahin et al., 2004), receptors like interleukin 6-receptor (IL-6R) (Müllberg et al., 1993; Müllberg et al., 1992) and tumor necrosis factor receptor (TNF-R1) (Reddy et al., 2000) and chemokines like fractalkine (CX3CL1) (Garton et al., 2001; Naus et al., 2006) and chemokine ligand-16 (CXCL16) (Gough et al., 2004). Several membrane-bound proteins are common substrates of different ADAMs (Edwards et al., 2008). Therefore, it is challenging to characterize ADAMs based on their substrate repertoire. Based on recent research advancements and proteomics studies, ADAM10 and ADAM17 are the two best-characterized sheddases in terms of their substrates and their regulatory proteins

(Lichtenthaler et al., 2018; Zunke & Rose-John, 2017). However, this list is now growing since more proteolytic ADAMs are now being characterized in terms of substrate cleavage and selectivity, e.g. in neurological disorders (Hsia et al., 2019) or in cancer malignancies (Arai et al., 2023). Amongst all the active ADAMs, ADAM17 and ADAM8 will be discussed in detail in the following sections.

1.2 ADAM17

ADAM17 is a ubiquitously expressed membrane-bound protease that was first identified as the TNF- α converting enzyme (TACE), releasing soluble and biologically active TNF- α from the cell surface (Black et al., 1997; Moss et al., 1997). As described above, ADAM17 shares structural similarities with its close relative ADAM10. In contrast to other ADAMs, the disintegrin domain is followed by a membrane-proximal domain (MPD) and the stalk region instead of an EGF-like domain (Figure 1) (Düsterhöft et al., 2013; Janes et al., 2005; Lambrecht et al., 2018). ADAM17 has been described as a canonical ‘full-time sheddase’ that is actively involved in the regulation of numerous physiological processes like cell signaling, tumorigenesis, proliferation, and inflammation via ectodomain shedding of diverse membrane-bound proteins such as TNF- α , EGFR ligands, and receptors like IL-6R (Blobel, 2005; Moss & Minond, 2017; Schumacher & Rose-John, 2022; Zunke & Rose-John, 2017). Studies have shown the impact of endogenous levels of TNF- α in cancer initiation and tumor progression (Balkwill, 2002). On the other hand, shedding of TNF receptors (TNF-R1 and 2) by ADAM17 releases sTNFR, which counteracts sTNF- α and blocks the pro-inflammatory response by cytokines (Bell et al., 2007). Upregulation of ADAM17 has been reported in cancer-associated malignancies. For instance, ADAM17 was highly expressed in primary colon carcinoma (Blanchot-Jossic et al., 2005), and ADAM17-mediated cleavage of CD44 induced the invasion in oral squamous cell carcinoma (Takamune et al., 2008). ADAM17 was also identified as one of the main lung cancer biomarkers in various lung cancer cells (Planque et al., 2009). Figure 2 shows some of the best-characterized substrates of ADAM17 and their functions in physiological and pathological events. ADAM17-mediated ectodomain shedding can be induced by stimulated G-protein coupled receptors (GPCR) or protein kinase C (PKC) via external stimuli like lipopolysaccharides (LPS), lysophosphatidic acid (LPA), or reactive oxygen species (ROS), or internal stimuli like calcium ion concentration (Blobel, 2005; Myers et al., 2009; Ohtsu et al., 2006). Additionally, the activity of ADAM17 can also be induced by activation of PKC via a synthetic activator, phorbol-12-myristat-13-acetate (PMA) which is a phorbol ester (Horiuchi, Le Gall, et al., 2007). In terms of chemokines, PMA induces ADAM17-mediated shedding of CX3CL1 (Garton et al., 2001). ADAM17 has been reported to enhance the metastasis of malignant hepatocytes by PMA-induced release of CX3CL1 (Sun et al., 2020). Further

ADAM17 is also reported to shed adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) (Tsakadze et al., 2006), vascular cell adhesion molecule-1 (VCAM-1) (Garton et al., 2003), and activated leukocyte cell adhesion molecule (ALCAM/CD166) (Bech-Serra et al., 2006) affecting the adhesive properties of cells. The shedding capacity of membrane-bound ADAM17 is not only limited to these substrates but at least 90 other substrates were identified *in vitro*, making it an important molecular switch (Düsterhöft et al., 2019; Pruessmeyer & Ludwig, 2009; Scheller et al., 2011).

Lack of functional ADAM17 in mice (ADAM17^{ΔZn/ΔZn}), resulting in embryonic lethality, developmental defects, and severe pathological effects suggested a significant role of ADAM17 in embryonic development (Peschon et al., 1998; Shi et al., 2003; Zhao et al., 2001). Since a complete knockout (KO) of ADAM17 in mice leads to premature death, conditional knockout models were generated to further comprehend the roles of ADAM17. A systemic knockout in murine myeloid cells prevented endotoxin shock-mediated death by reducing TNF- α levels in the serum, suggesting ADAM17 as a major treatment target in pathologies caused by TNF- α shedding (Horiuchi, Kimura, et al., 2007). Since then, many other conditional knockout mice have been generated targeting different tissue systems that proved the significance of metalloprotease in development and pathologies (Rahn & Becker-Pauly, 2022; Weber & Saftig, 2012; Zunke & Rose-John, 2017). Additionally, the hypomorphic ADAM17 mouse model (ADAM17^{ex/ex}), which had reduced levels of ADAM17 by 95% was viable but displayed eye, skin, and heart defects due to hampered shedding of EGFR ligands causing failed EGFR signaling and intestinal inflammation (Calligaris et al., 2021; Chalaris et al., 2010). Altogether, ADAM17 is one of the central players in the complex network of cell signaling and modulates several cellular events.

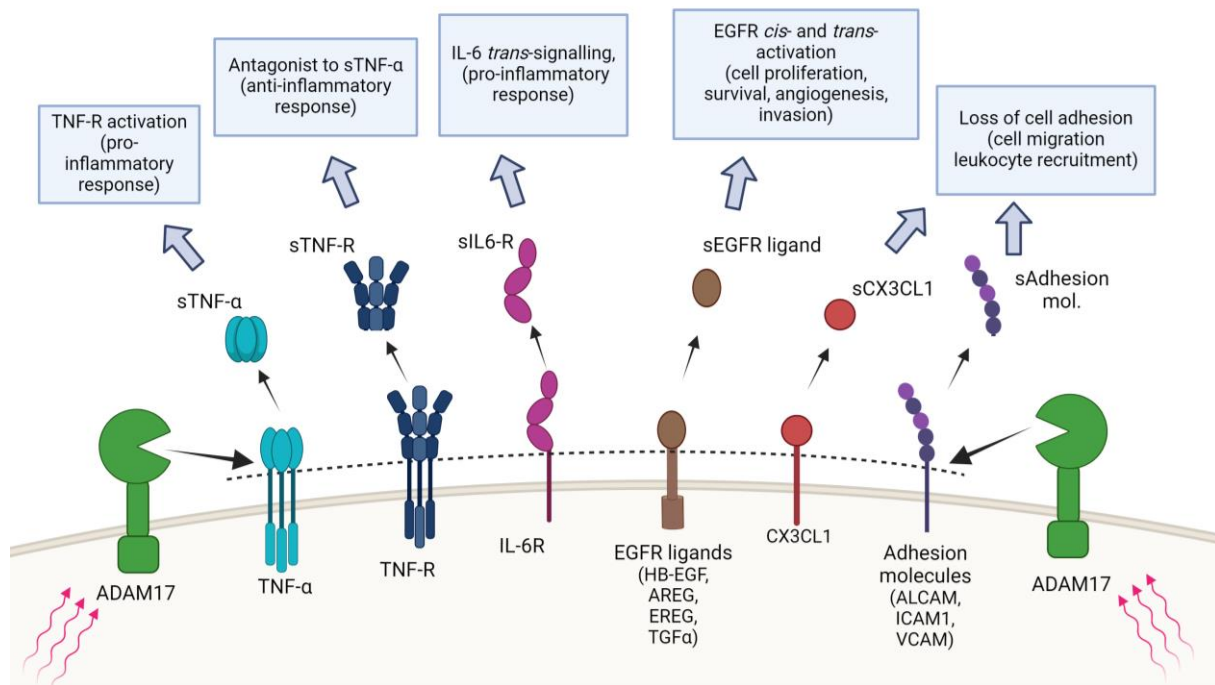


Figure 2: ADAM17 in physiological and pathological processes

ADAM17 participates in physiological and pathophysiological processes via shedding of a variety of substrates including cytokines, cell surface receptors, adhesion molecules, and growth factors to induce or modulate cell signaling. Examples of ADAM17-substrate relationships and subsequent effects are shown prototypically. The picture is adapted and created from (Düsterhöft et al., 2019; Rahn & Becker-Pauly, 2022; Scheller et al., 2011)

1.2.1 ADAM17 in EGFR signaling mediated pathologies

EGFR, also known as ErbB1/HER1 is one of four members of the ErbB family of tyrosine kinase receptors and is a glycosylated transmembrane protein. Structurally, it consists of an extracellular domain, a TM-domain, and an intracellular domain with tyrosine kinase activity. The EGFR is crucial for initiating the signaling in epithelial cells and tumors of epithelial cell origin (Herbst, 2004). Most tumors like breast cancer, colon cancer, ovarian cancer, and head-and-neck cancer express high levels of EGFR, which is associated with aggressive proliferation and invasion (Carpenter & Cohen, 1979; Salomon et al., 1995). EGFR plays a key role in embryonic development. EGFR deficiency in mice leads to impaired epithelial development in organs like skin, gastro-intestinal tract, and lungs, combined with death within 8 days of birth (Miettinen et al., 1995). The loss-of-function studies performed in mice revealed novel functions of EGFR in major organ development like skin, brain, heart, bone, lungs, and liver (Sibilia et al., 2007; Sibilia et al., 2003). Furthermore, the EGFR knockout animals phenocopy the ADAM17 deficient mice in terms of multiple organ defects, suggesting once again the importance of ADAM17 and EGFR alliance (Chalaris et al., 2010; Miettinen et al., 1995).

EGFR ligands are synthesized as type-1 membrane proteins that are further processed by ectodomain shedding via ADAM proteases for the release of sEGFR ligands which consequently bind to the EGFR (Sahin et al., 2004). A total of seven EGFR ligands can bind to the EGFR after the proteolytic release of their soluble forms as a prerequisite to initiate EGFR signaling. ADAM17 is reportedly the major sheddase of most of the EGFR ligands (Blobel et al., 2009). Transforming growth factor- α (TGF- α), heparin-binding-epidermal-growth factor (HB-EGF), amphiregulin (AREG), epiregulin (EREG), and epigen are known substrates of ADAM17. On the other hand, ADAM10 which is closely related to ADAM17 is responsible for the shedding of BTC (betacellulin) and EGF (epidermal-growth-factor) (Sahin & Blobel, 2007; Sahin et al., 2004). Upon release of soluble EGFR ligand via ectodomain shedding, the ligand binds to the EGFR and the receptor dimerizes leading to activation of the receptor in an autocrine or paracrine manner (Figure 3) (Schlessinger, 2002). Activation of the EGFR by its ligand leads to dimerization of the receptor and subsequent activation of intracellular signaling cascades involved in a wide range of cellular processes including differentiation, cell proliferation, invasion, metastasis, and angiogenesis (Gschwind et al., 2003; Jeppe Knudsen et al., 2014; Singh et al., 2016).

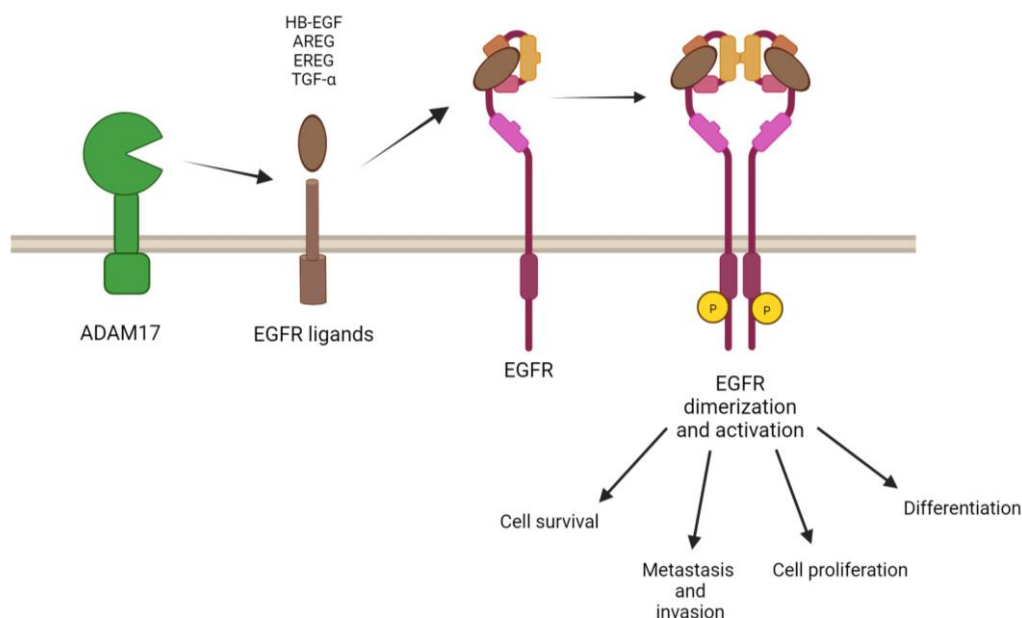


Figure 3: Schematic representation of ADAM17 function in EGFR signaling

The membrane-bound metalloprotease ADAM17 cleaves off the extracellular part of the membrane-bound form of EGFR-ligands like AREG, HB-EGF, EREG, or TGF- α (brown). The released soluble EGFR ligands (brown oval) bind to the EGF receptor and induce the dimerization and activation of the receptor (pink and yellow). This initiates intracellular signaling pathways that regulate a variety of cellular processes.

By processing most of the ligands of the EGFR, ADAM17 is one the most important regulators of EGFR signaling. As mentioned above, the hypomorphic ADAM17 mice showed impaired EGFR signaling as a result of inefficient shedding of TGF- α and amphiregulin causing intestinal inflammation and impaired milk duct formation (Chalaris et al., 2010). ADAM proteases have

partially overlapped substrate specificities. However, shedding of EGFR-ligands in wild-type and triple knockout ADAM9/12/15^{-/-} cells showed the major involvement of ADAM17 in PMA-stimulated shedding of AREG, HB-EGF, and TGF- α . Additionally, the stimulated shedding of AREG, HB-EGF, TGF- α , and EREG was strongly decreased in the absence of ADAM17, but not in the absence of ADAM10 (Sahin et al., 2004). Moreover, the shedding was strongly increased in the presence of phorbol ester (PMA), suggesting ADAM17-mediated shedding of epigen (Sahin & Blobel, 2007). In addition to that, the analysis of constitutive and PMA-induced shedding of CXCL1 and epigen in Cos-7 cells showed that ADAM17-mediated shedding was inhibited only by the hydroxamate inhibitor GW280164X (GW), but only constitutive shedding of CXCL1 was inhibited by the ADAM10 specific inhibitor GI254023X (GI) (Ludwig et al., 2005; Sahin & Blobel, 2007).

In vitro and whole animals, studies have also shown that lack of ADAM17 is associated with impaired shedding of AREG, HB-EGF, and TGF- α resulting in eye, hair, and skin defects (Peschon et al., 1998; Sunnarborg et al., 2002). Mice lacking HB-EGF die before weaning or within the first year of life and display similar heart valve defects as mice deficient in ADAM17 and EGFR (Jackson et al., 2003). Moreover, in ovarian cancer tissues, a positive correlation between ADAM17 and HB-EGF was observed due to the upregulation of both proteins (Tanaka et al., 2005). ADAM17 plays an essential role in epithelial-stromal crosstalk. This drives mammary development by the proteolytic release of AREG from epithelial cells and activation of EGFR on stromal cells, showing the scope of the paracrine signaling pathway (Sternlicht et al., 2005). ADAM17 has been reported to play a crucial part in receptor crosstalk upon its activation by the stimulation of G-protein coupled receptors (GPCR), leading to substrate shedding and subsequent phosphorylation of EGFR bound to the ligand which finally initiates a signaling cascade (Gooz, 2010). In human colon adeno carcinoma cells (HCA-7), endogenous levels of amphiregulin in the supernatant were only reduced by inhibiting ADAM17 with broad spectrum metalloprotease inhibitor Marimastat and ADAM17 inhibitor, GW (Schmidt et al., 2018).

1.2.2 Regulation of ADAM17

The pivotal role of ADAM17 in fundamental biological processes such as pathologies and homeostasis mediated by ectodomain shedding requires tight regulation of the protease. (Zunke & Rose-John, 2017). When dysregulated, ectodomain shedding contributes to cell pathophysiological conditions (Dreymueller et al., 2015; Hsia et al., 2019; Jones et al., 2016; Murphy, 2008). Therefore, the regulation of a protease or a proteolytic pathway is a multidimensional process ranging from transcriptional to post-translational control in terms of protein synthesis, maturation and PTMs, trafficking, activation, and inhibition. At the

transcriptional level, nuclear factor-kappa B (NF- κ B) has been shown to upregulate ADAM17 mRNA expression upon induction with TNF- α (Takamune et al., 2008). Furthermore, the inhibition of NF- κ B via activation of Ron receptor tyrosine kinase leads to decreased ADAM17 levels in murine alveolar macrophages (Nikolaidis et al., 2010). Post-transcriptionally, the targeted inhibition of ADAM17 mRNA silenced the ADAM17 gene, resulting in decreased protein expression and hence reduced ADAM17-mediated cancer invasion (Lu et al., 2013; Sun et al., 2013; X. W. Yang et al., 2014). As mentioned before, the activity of ADAM17 can be induced via external and internal stimulation of GPCRs and PKC, which suggests the crosstalk between these proteins is important to regulate ADAM17 activity (Calligaris et al., 2021; Zunke & Rose-John, 2017). Several studies demonstrated the activation of ADAM17 via the phosphorylation of its cytosolic domain by PMA-mediated stimulation of PKC or Mitogen-activated protein kinases (MAPK) (Müllberg et al., 1992; Xu & Derynck, 2010). However, it was later shown that the cytoplasmic domain and its phosphorylation are not essential for ADAM17 activity (Le Gall et al., 2010; Schwarz et al., 2013). In addition, other domains of ADAM17 are also reported to play important roles in the regulation of its activity. The pro-domain of ADAM17 keeps the molecule in a switched-off mode until it is proteolytically removed by furin or furin-like proprotein convertases (Wong et al., 2016; Wong et al., 2015). The disintegrin domain interacts with the integrin ($\alpha 5 \beta 1$) regulating the activity of ADAM17-mediated cell adhesion and migration (Bax et al., 2004; Calligaris et al., 2021). The MPD has also been reported to keep the conformation of ADAM17 in an open or closed form. The cysteine residues in the MPD undergo isomerization caused by protein-disulfide isomerases (PDI) converting the less structured open and active conformation into a well-defined closed and inactivated structure (Düsterhöft et al., 2013; Willems et al., 2010). Furthermore, the MPD is also reported to interact with phosphatidylserines (PS), which is thought to regulate the positioning of the protease at the membrane to gain shedding activity (Sommer et al., 2016). In addition to these mechanisms, interaction with inactive rhomboid proteins (iRhom1 and iRhom2), endogenous inhibitors, and glycosylation have been shown to regulate ADAM17 maturation and activity. (Adrain & Freeman, 2012; Adrain et al., 2012). Further, a study also addressed the release of ADAM17 in exosomes via stimulation of cells through lipopolysaccharides leading to a downregulation of ADAM17 on the cell surface. The released exosomes were reported to exhibit ADAM17-mediated shedding of TGF- α upon incubation with cells expressing TGF- α (Groth et al., 2016).

1.2.2.1 Regulation by iRhoms

As the name suggests, iRhoms are catalytically inactive homologs of rhomboid intramembrane serine proteases lacking essential catalytic residues and therefore known as pseudoproteases (Lemberg & Freeman, 2007). First identified in *Drosophila*, the active rhomboids are

responsible for EGFR signaling (Urban et al., 2001; Urban et al., 2002; Wasserman et al., 2000). On the other hand, the inactivated rhomboids present in the ER completely stall the EGF processing and direct the protein toward ER-associated degradation (ERAD) (Zettl et al., 2011). In mammals, two iRhoms, iRhom1 and iRhom2 are mostly located in the ER and reported to perform a different function than ERAD (Adrain & Freeman, 2012; Lemberg & Freeman, 2007). However, ER-associated death has also been observed in mammals (Düsterhöft et al., 2019; Lee et al., 2015). As reviewed recently, together with the studies in various iRhom mouse models and the roles of inactive rhomboids in EGFR signaling, iRhoms are also reported to regulate EGFR signaling in mammals (Burzenski et al., 2021).

iRhoms in maturation and trafficking of ADAM17

From a regulatory point of view, iRhom1 and 2 are under discussion to regulate membrane-bound ADAM17 in terms of its trafficking from the ER to the Golgi, its maturation, and its activation by furin as well as its shedding activity (Adrain et al., 2012; Christova et al., 2013; Dulloo et al., 2019; Maretzky et al., 2013). Figures 4A and B demonstrate the scenario in the absence and the presence of iRhoms and its impact on ADAM17-mediated shedding.

Further studies demonstrated that iRhom1/2 single knockout mice were viable and fertile but iRhom1/2 double knockout mice phenocopied ADAM17 knockout mice and died perinatally (Li et al., 2015). In the absence of iRhom1, the developmental defects were not observed but the maturation of ADAM17 was compromised in the brain (Li et al., 2015). This suggested that iRhom1 and iRhom2 are expressed in a tissue-dependent manner and are noticed to exhibit overlapping functions pertaining to the trafficking and maturation of ADAM17 (Issuree et al., 2013; Li et al., 2015). On the contrary, another study showed that iRhom1 single knockout mice showed severe multiple organ defects, and iRhom1/2 double knockout mice displayed early embryonic lethality (Christova et al., 2013). Additionally, the mouse model created by Li et al. in 2015 was found to have a malfunction, in terms of partial retainment of the iRhom1 function due to incomplete deletion of the target gene (Hosur et al., 2020; Li et al., 2015). In humans and mice, iRhom1 is expressed in most of the cells, whereas iRhom2 is predominantly expressed in myeloid cells. Hence, in macrophages, a reduced stimulated shedding of TNF- α was observed exclusively in the absence of iRhom2 (Adrain et al., 2012; Li et al., 2015; McIlwain et al., 2012; Siggs et al., 2012). Evidently, for TNF- α , iRhom2 appears to play the role of a major regulator of ADAM17 for its forward trafficking and its activation (Adrain et al., 2012; Dulloo et al., 2019). In addition to that, in primary mouse macrophage culture and embryonic fibroblasts, ADAM17 has been implicated in the exclusive stabilization of iRhom2 but not iRhom1 (Weskamp et al., 2020). Depending on the expression profile, iRhom1 and iRhom2 are known to participate in the regulation of ADAM17. However, little was known about the role of iRhom1 until it was recently found out that iRhom1 is broadly expressed in the

murine brain and governs the maturation and trafficking of ADAM17 (Tushaus et al., 2021). Thus, for the regulation of membrane-bound ADAM17, iRhom1 and 2 are indispensable.

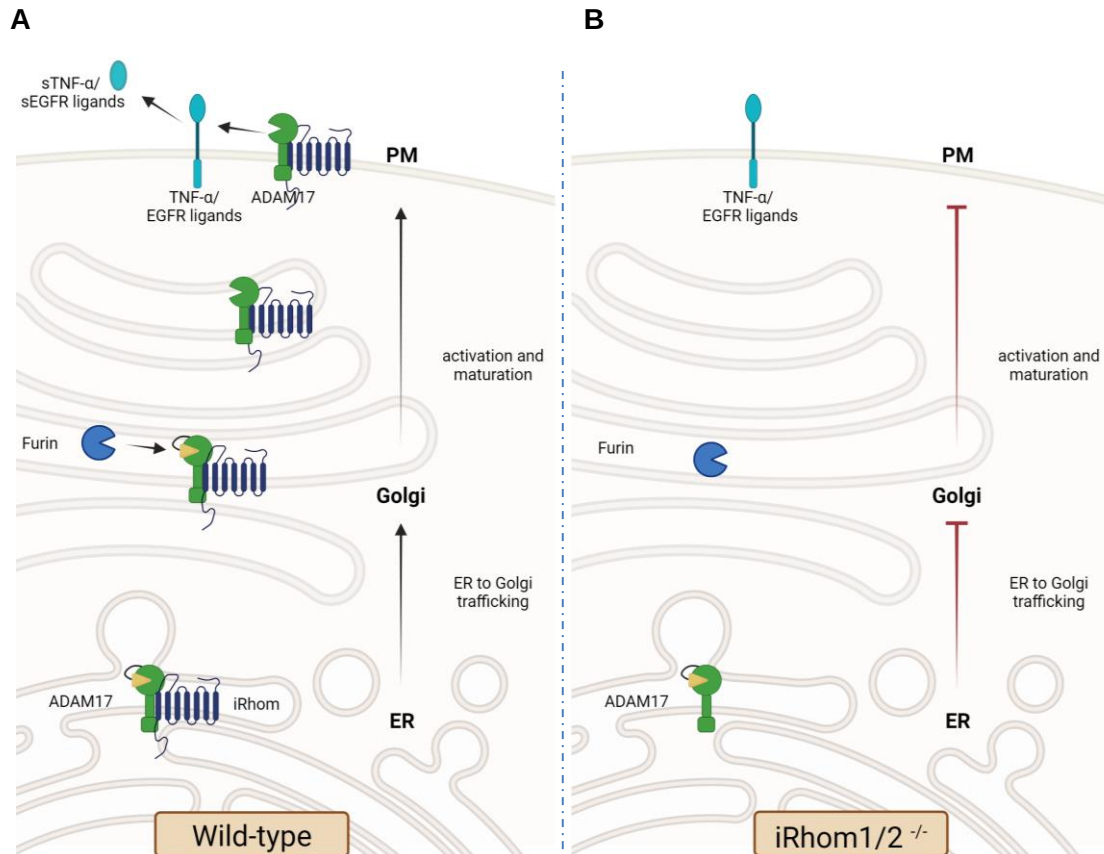


Figure 4: iRhoms in maturation and trafficking of ADAM17

The inactive rhomboid proteases iRhom1 and iRhom2 play key roles in ADAM17 maturation and trafficking. (A) iRhoms participate in the anterograde trafficking of pro-ADAM17 from the ER to the Golgi, where the maturation and activation of ADAM17 occurs. Mature, membrane-bound ADAM17 is then trafficked to the plasma membrane (PM) where it sheds its substrates (shown in wild-type condition). (B) In the absence of iRhoms, this process is abolished, and ADAM17-mediated activity is stalled (shown in the iRhom1/2 knock-out condition). Adapted from (Adrain & Freeman, 2012).

The structural determinants of the iRhom-ADAM17 interaction that regulate the transport, maturation, and activity of ADAM17 are only partially understood. It was found that the interaction of the transmembrane helix (TMH) 1 of iRhoms and the TM-domain of ADAM17 is crucial for efficient binding of the complex (Cavadas et al., 2017; Li et al., 2017). In addition, iRhoms possess two other functionally and structurally important regions: a long cytosolic N-terminus and a large luminal iRhom homology domain (IRHD) between TMH1 and 2 (Lemberg & Freeman, 2007). The latter is shown to be essential for the transport of ADAM17 from the ER to the Golgi and thus for ADAM17 maturation, but not for the interaction between the two proteins (Düsterhoft et al., 2021).

iRhoms in ADAM17-mediated activity

The role of iRhoms is not only restricted to the maturation and trafficking of ADAM17 but they are also actively involved in the regulation of its shedding activity, thereby indirectly controlling signaling cascades. According to the mice models, like their active counterparts, iRhoms have a crucial role in the regulation of EGFR signaling. The iRhom1/2 double knockout (iRhom1/2^{-/-}) mice resemble ADAM17 and EGFR knockout mice, and the EGFR signaling is significantly abolished except for the signaling via the ADAM10 substrate BTC, hence proving iRhom1/2 as critical components of the EGFR pathway (Li et al., 2015). Predominantly, iRhom2 has been rigorously investigated in association with ADAM17-mediated shedding of its substrates from the cell surface. Further studies in mouse embryonic fibroblasts (mEFs) suggested that under stimulated conditions, iRhom2 is essential for the shedding of HB-EGF, AREG, and EREG, but not for TGF- α and the ADAM10 substrate BTC (Maretzky et al., 2013). Additionally, the deletion of the entire N-terminal cytoplasmic domain of iRhom2 interrupted the shedding of iRhom2-dependent EGFR ligands (Maretzky et al., 2013). On the other hand, the interface between ADAM17 and iRhom2 TM-helices is noted to be an important criterion for ADAM17 activation and shedding function, since individual mutations in the TM-domain of ADAM17 lead to inefficient stimulated shedding of EREG, but not TGF- α (Li et al., 2017). Taken together, this indicated that along with the maturation and trafficking of ADAM17, the substrate selectivity of ADAM17 is also controlled by iRhom1 and iRhom2 (Düsterhöft et al., 2019; Geesala et al., 2019). Furthermore, a recent study identified MEGF10 (multiple EGF-like domain factor 10) as an iRhom1-dependent substrate of ADAM17 in mice brains (Tushaus et al., 2021). Collectively, both the iRhoms can take over each other's role and act in a tissue-dependent manner (Burzenski et al., 2021).

The tight iRhom2/ADAM17 complex formation prevents ADAM17 from lysosomal degradation (Grieve et al., 2017). Studies have highlighted the importance of the cytosolic part of N-terminus of iRhom2 for the alteration in this conformation through phosphorylation of the cytosolic upon stimulation via PMA or GPCRs. This reaction induces the separation of iRhom2 and ADAM17 and renders the latter to access its substrate efficiently (Cavadas et al., 2017; Grieve et al., 2017) (Figure 5). However, this phosphorylation does not play a significant role in the maturation and trafficking of ADAM17 (Grieve et al., 2017).

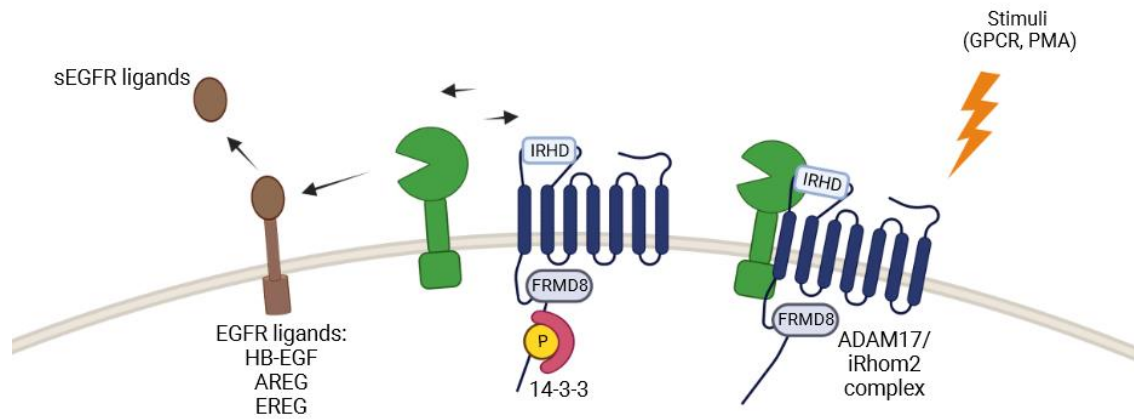


Figure 5: Regulation of ADAM17 activity by iRhom2

In response to stimuli like PMA or GPCRs, the N-terminal cytosolic tail of iRhom2 gets phosphorylated. iRhom2 then recruits 14-3-3 proteins causing the disassociation of the ADAM17 and iRhom2 complex, which makes ADAM17 more accessible to its substrates. Adapted from (Cavadas et al., 2017; Grieve et al., 2017).

For the regulatory machinery for ADAM17 to function properly, several components, such as adaptor proteins, are needed. For example, phosphorylation recruits 14-3-3 protein to the N-terminal tail of iRhom2 for the weakening of the iRhom2/ADAM17 complex, to start the shedding cascade (Cavadas et al., 2017; Grieve et al., 2017). Furthermore, another adaptor protein FRMD8/iTAP (a protein containing FERM domain/ iRhom Tail associated protein) was identified to bind to the N-terminal tail of iRhom and markedly maintain the levels of iRhom and ADAM17 *in vitro*. However, a deeper understanding of the mechanism is needed (Kunzel et al., 2018; Oikonomidi et al., 2018). Further studies have suggested that the extracellular juxtamembrane domains of iRhom2 and ADAM17 are important for the substrate specificity in addition to the TMH interaction (Tang et al., 2020). This was supported by the report that the highly conserved iRhom homology domain (IRHD) regulates the ADAM17-mediated release of TNF- α and EGFR-ligands such as TGF- α , HB-EGF and AREG from the cell surface (Düsterhoft et al., 2021; Kahveci-Türköz et al., 2023). Taken together, the close interaction between ADAM17 and iRhom1/2 could be an interesting way to the indirect inhibition of ADAM17 activity by targeting iRhoms.

1.2.2.2 Regulation by inhibition

The engagement of ADAM17 in physiological and pathological conditions may require pharmacological regulation of ADAM17, which could be achieved by the inhibition of the molecule via specific endogenous or synthetic inhibitors. The homology of ADAM17 and ADAM10 proves to be challenging for the specific inhibition. However, in the past many such molecules came into light that showed promising inhibition of ADAM17, blocking the specific

pathways. Out of four identified tissue inhibitors of metalloprotease (TIMPs), TIMP-3 inhibits ADAM17 at the cell surface endogenously (Amour et al., 1998). Additionally, ADAM17-mediated EGFR signaling via shedding of HB-EGF is also compromised in the presence of TIMP-3 and prevents the risk of liver cancer during hepatitis infection (Jackson et al., 2017; Murthy et al., 2010). Further, the recombinant pro-domain of ADAM17 has displayed highly specific inhibition of ADAM17 activity both *in vitro* and *in vivo* (Wong et al., 2016). This was further substantiated when the application of the recombinant pro-domain of ADAM17 blocked metastasis and necroptosis of mouse endothelial cells (Bolik et al., 2022). Furthermore, overexpression of the ADAM17 pro-domain promoted neuroregeneration in brain injury blocking the EGFR signaling, by specifically inhibiting the ADAM17 activity against TGF- α (Geribaldi-Doldán et al., 2018). Several ADAM17-specific monoclonal antibodies such as D1(A12) and D8P1C1 have been shown to promote anti-tumorigenic effects by inhibiting EGFR signaling (Caiazza et al., 2015; Saha et al., 2022; Tape et al., 2011).

1.2.2.3 Regulation by glycosylation

Apart from the post-translational processing of ADAM17 by proteolytic removal of the pro-domain and through phosphorylation, another PTM in the regulation of ADAM17 is the glycosylation of the molecule. The experiments performed on recombinant ADAM17 obtained from mammalian and insect models showed significant variation in the glycosylation complexities (Chavaroche et al., 2014). The heavily glycosylated mammalian ADAM17 had a higher proteolytic activity towards its substrates as compared to ADAM17 from insect cells (Chavaroche et al., 2014). In the same study, the inhibition of differentially glycosylated ADAM17 was significantly different by applying exosite or non-zinc binding inhibitors (Chavaroche et al., 2014). This implies that glycosylation adds another layer to the activation and inhibition of ADAM17. In addition to correct glycosylation of ADAM17, the glycan addition pattern of the ADAM17 substrates can also result in variation in shedding capability. Proteolysis can be prevented by site-specific glycosylation of the substrates; for example, by site-specific O-glycosylation, the shedding of TNF- α by ADAM17 was inhibited (Goth et al., 2015). Interestingly, the Golgi-acquired glycosylation of iRhom2 is also proven important for the interaction with ADAM17 and the latter's cell surface presentation (Düsterhoft et al., 2021). This in turn regulates receptor-mediated signal transduction facilitated by ADAM17 (Düsterhoft et al., 2021).

1.3 ADAM8

ADAM8 was first discovered as a cell surface antigen on mice monocytes and is also called cell surface antigen MS2 or CD156 (Yoshida et al., 1990). Later, it was identified as an active metalloprotease in osteoclasts (Choi et al., 2001). It is localized at the plasma membrane and throughout the secretory pathway (Schlomann et al., 2002). ADAM8, like its other relatives, consists of a typical ADAM family domain composition: the inhibitory pro-domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain followed by a cytoplasmic C-terminal domain. However, ADAM8 has a slightly different consensus cleavage sequence (RETR instead of the dibasic sequence RXK/RR), which is necessary for furin-like PC cleavage (Barr, 1991; Hall et al., 2009; Schlomann et al., 2002). Thus, removal of the pro-domain is independent of furin or furin-like PCs, and ADAM8 is autocatalytically activated after oligomerization (Hall et al., 2009; Schlomann et al., 2002). Experimentally, in the murine ADAM8, a proteolytically inactive mutant was generated by exchanging the glutamate residue in the zinc-binding domain at position 330 with glutamine (E330Q/EQ-ADAM8). The proteolytic removal of the pro-domain was completely abolished due to this mutation (Schlomann et al., 2002). The multimerization is supported by the disintegrin, cysteine-rich, and EGF-like domains, which often results in two isoforms of ADAM8; the 60 kDa remnant form attached to the cell surface and a 30 kDa soluble form released into the ECM (Schlomann et al., 2015; Schlomann et al., 2002). In addition to the active ADAM8, the remnant form is also known to interact with integrins and perform cell adhesion functions (Rao et al., 2006). Furthermore, the soluble ADAM8 consisting of the metalloprotease domain, was reported to be proteolytically active by cleavage of myelin basic protein (MBP) and CD23. This activity was inhibited by batimastat (BB-94), BK1361, and 1,10-phenanthroline (Schlomann et al., 2015; Schlomann et al., 2002). Later studies also confirmed the proteolytic roles of soluble ADAM8 in the ECM (Zack et al., 2009).

Unlike ADAM17, ADAM8 is selectively expressed in the central nervous system, in osteoclasts, in the epithelial lining of the lungs, and weakly in immune cells (Choi et al., 2001; King et al., 2004; Schlomann et al., 2000; Yoshiyama et al., 1997). The targeted deletion of ADAM8 in mice revealed the expression profile of ADAM8 during developmental and adult stages. During embryonic development, ADAM8 is expressed in the developing brain, thymus, lymphatic-vessels, and bone cartilage (Kelly et al., 2005). Furthermore, in adult mice, the expression was restricted to the epithelia of salivary glands, respiratory bronchioles, and kidney as well as the lymphatic organs spleen and thymus (Kelly et al., 2005). However, the knockout of ADAM8 did not show any developmental defects or adverse phenotypes in mice (Kelly et al., 2005).

1.3.1 Proteolytic and non-proteolytic functions of ADAM8

Although ADAM8 has been identified as a non-essential protease for development, it is highly expressed in various pathologies such as inflammation and cancer (Koller et al., 2009). ADAM8 exhibits an overlapping substrate spectrum with ADAM10 and ADAM17; including L-selectin, Amyloid precursor protein (APP), and tumor necrosis factor receptor 1 (TNF-R1) (Bartsch et al., 2010; Conrad et al., 2019; Gómez-Gaviro et al., 2007). *In vitro*, screening of peptides from substrates of other ADAMs revealed further substrates such as TGF- α , TNF- α , and CD23 (Naus et al., 2006). The ADAM8-mediated shedding of the TNF receptor is induced by the cytokine TNF- α , creating a neuroprotective feedback loop. The access of TNF- α causes the TNF-R1-induced ADAM8 transcription, which in turn cleaves the cell surface TNF-R1, releasing soluble TNF-R1 that seizes TNF- α (Bartsch et al., 2010). This supports the previous finding that TNF- α triggers the upregulation of ADAM8 in the neuronal cells affected by neurodegeneration (Schlommann et al., 2000). In experimental models, ADAM8 was noted to modulate leukocyte recruitment and migration in asthma, as ADAM8 knockout leukocytes were less infiltrating (Naus et al., 2010). Additionally, in patients with acute respiratory distress syndrome (ARDS), the proteolytic activity of ADAM8 is directly proportional to high fatality (Conrad et al., 2022). ADAM8 is reported to affect the migration and rolling of leukocytes during inflammation by shedding P-selectin glycoprotein ligand-1 (PSGL-1) (Domínguez-Luis et al., 2011). Increased levels of ADAM8 have been found in aggressive pancreatic cancer, inducing invasion due to proteolytic activity, which correlated with poor patient survival rates (Valkovskaya et al., 2007). Other studies have conducted tissue microanalysis and detected increased ADAM8 expression in lung cancer cells, suggesting it as a diagnostic biomarker (Hernández et al., 2010; Ishikawa et al., 2004). In human primary brain cells, invasiveness and migration are associated with the proteolytic activity of upregulated ADAM8 (Wildeboer et al., 2006). ADAM8 has been additionally associated with poor patient outcomes in several other cancers, like lung cancer, glioma, and colorectal cancer (He et al., 2012; Ishikawa et al., 2004; Z. Yang et al., 2014). In pancreatic ductal adenocarcinoma (PDAC), ADAM8 is essential for the release of MMP9, which is an important metalloprotease for tumor progression (Cook et al., 2022; Mierke, 2023).

Besides its proteolytic functions, studies have also shown the non-proteolytic functions of ADAM8 in certain signaling pathways. As mentioned earlier, ADAM8 interacts with integrins through its disintegrin domain, thereby activating integrin signaling pathways by phosphorylation of focal adhesion kinase (FAK), mitogen-activated kinase (MAPK) and extracellular regulated kinase (ERK1/2) (Awan, Babendreyer, Mahmood Alvi, et al., 2021; Conrad et al., 2018; Dong et al., 2015). In a recent study, ADAM8 was reported to upregulate the expression of HB-EGF, as ADAM8 knockdown resulted in significantly decreased HB-EGF expression (Liu et al., 2022). ADAM8 was observed to non-proteolytically mediate a positive

feedback loop between malignant glioblastoma and tumor-associated macrophages (TAMs) via HB-EGF/EGFR signaling (Liu et al., 2022). Of note, HB-EGF is not a shedding substrate of ADAM8 (Schlomann et al., 2015). Further, the C-terminal domain of ADAM8 has been reported to induce downstream signaling via phosphorylation of ADAM8 and its interaction with adaptor proteins via SH3 motifs (Kleino et al., 2015). In the same line, in aggressive brain cancer angiogenesis, ADAM8 non-proteolytically modulates the intracellular signaling by regulating osteopontin (OPN) expression via its disintegrin and cytoplasmic domain (Li et al., 2021).

1.3.2 Regulation of ADAM8

In contrast to the complex regulation of ADAM17, which has been extensively studied, the underlying mechanisms for ADAM8 are only partially understood. Nonetheless, few studies shed light on possible factors including stimulators like PMA, bacterial lipopolysaccharide (LPS), and ADAM inhibitors, involved in the regulation of ADAM8 in inflammation. In human neutrophils, PMA promotes the translocation of ADAM8 from the neutrophil granules to the cell surface, which results in enhanced cleavage of L-selectin and also the release of the ectodomain of ADAM8 (sADAM8) (Gómez-Gavero et al., 2007). This suggests that targeting ADAM8 to prevent excessive shedding of L-selectin will aid in controlled neutrophil migration (Gómez-Gavero et al., 2007). Additionally, in inflammation, the expression of ADAM8 gets upregulated in the presence of LPS (Kataoka et al., 1997). Moreover, LPS-induced hepatocytes and endothelial cells express high levels of ADAM8, which correlates with increased levels of chemokines and cytokines. These can be downregulated by treating the cells with broad-spectrum inhibitors like BB-94 and peptidomimetic cyclic inhibitor (BK-1361) or knocking out ADAM8 (Awan, Babendreyer, Wozniak, et al., 2021). By treating mice with BK1361, the multimerization and activation of ADAM8 can be inhibited (Schlomann et al., 2015). In acute lung inflammation, this inhibition of ADAM8 decreases the neutrophil influx into the lungs (Conrad et al., 2022).

In addition to the above-mentioned regulations, N-glycosylation at multiple sites is noted to be significant for the regulation of processing, activation, and cellular localization of ADAM8 (Srinivasan et al., 2014).

1.3.3 Proteolytic generation of sADAM17 via ADAM8

The function of each protease is embedded in a higher regulatory network that integrates complex interactions with other proteases as well as inhibitors, the so-called protease web (Fortelny et al., 2014). A so far rather less explored direct interplay between different proteases

is that membrane-bound sheddases are also shed themselves, thus extending their proteolytic activity from the plasma membrane further into the ECM. Such a regulatory proteolytic network has been described for meprin β and ADAM10/17. On the one hand, the inactive pro-form of meprin β is shed by ADAM10 and ADAM17 (Hahn et al., 2003; Herzog et al., 2014; Wichert et al., 2017). On the other hand, once activated, mature meprin β is not shed anymore but can fine-tune ADAM10/17 activity at the plasma membrane by pro-domain cleavage N-terminal to the furin cleavage site, resulting in enhanced ADAM activity (Wichert et al., 2019).

In the same line, the shedding of ADAM10 by ADAM9 and ADAM15 or by autoproteolysis has been described (Suh et al., 2013; Tousseyn et al., 2009; Wichert et al., 2019), but functional consequences have been discussed mainly in terms of impaired ectodomain shedding or toward potential signaling of the released intracellular C-terminal fragment. In the case of ADAM17, elevated levels of the protease have been reported in serum samples from patients with lung cancer and psoriasis (Gül et al., 2022; Planque et al., 2009). Moreover, ADAM17 activity has been detected in the blood plasma of patients with vasculitis and Alzheimer's disease (Bertram et al., 2015; Sun et al., 2014) as well as in cerebrospinal fluid (Conrad et al., 2017; Jiang et al., 2011). However, none of these studies addressed the origin of the demonstrated presence of ADAM17.

According to one study, the knockout of ADAM8 in MDA-MB-231 cells resulted in significantly decreased ADAM17 levels in the medium as detected by soluble receptor array and ELISA (Scharfenberg et al., 2020). The proteolytic release of the ADAM17 ectodomain was further investigated in ADAM10/17-/- HEK293T cells using murine ADAM17 and ADAM8 expression constructs, demonstrating for the first time that the ~70 kDa ectodomain of ADAM17 can be also proteolytically released by the identified sheddase ADAM8 (Scharfenberg et al., 2020).

Considering the N-terminomics approach using recombinant ADAM17 ectodomain, initial shedding assays, and *in vitro* validation of novel, candidate substrates this study provided proof of concept that shed ADAM17 may exhibit additional functions within the protease web as a soluble enzyme, which is rather a feature of 'part-time' sheddases (Figure 6).

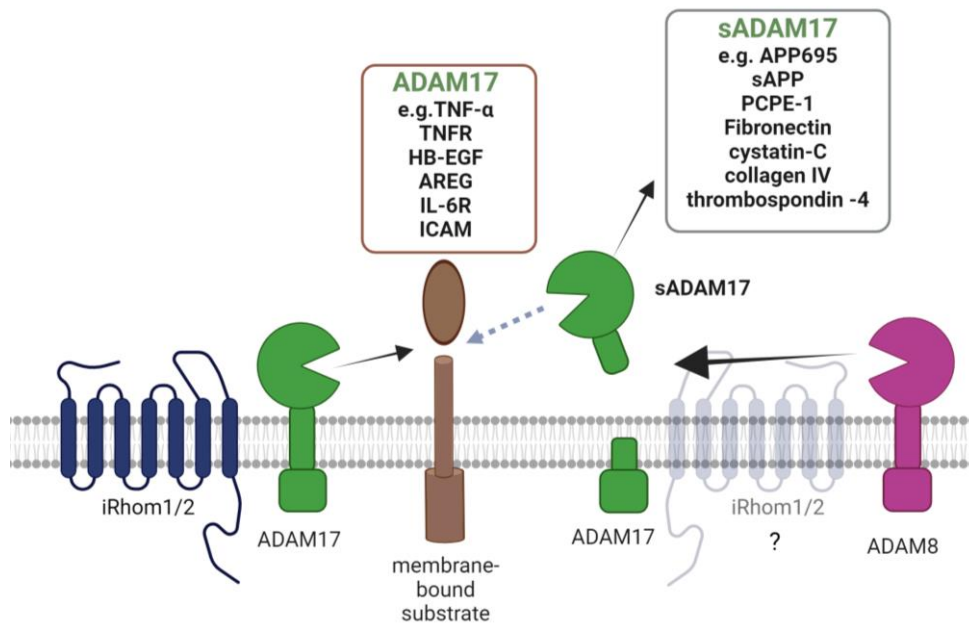


Figure 6: Part-time sheddase model of ADAM17

The shedding activity and specificity of membrane-bound ADAM17 are regulated by iRhoms. From the ~90 identified substrates; some well-characterized examples are shown. ADAM17 itself can get shed by ADAM8, releasing soluble ADAM17 in the ECM. *In vitro* studies using the recombinant ADAM17 ectodomain have revealed potential so far underestimated functions of ADAM17 as a soluble enzyme, suggesting that ADAM17 might be a “part-time” sheddase.

1.4 ADAM17 and ADAM8 in breast cancer

According to a recent global statistical analysis, with almost 12% of cases, breast cancer is the most commonly diagnosed cancer and a prominent cause of death in women (Sung et al., 2021). Breast cancer is highly heterogeneous in terms of differential gene expression and phenotypes. The early studies identified subtypes of cancer based on gene expression profiling (Perou et al., 2000). The subtypes are generally defined based on the expression of tumor-associated receptors; Estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER-2), and Ki67 (Hergueta-Redondo et al., 2008). Depending upon the expression of the mentioned receptors, an additional subtype of breast cancer was defined as triple-negative breast cancer (TNBC), that is an ER, PR, and HER-2 negative cancer (Carey et al., 2007; Carey et al., 2006). TNBC, itself is a complex cancer type with further six subtypes: basal-like 1/2 (BL1 and BL2), immunomodulatory (IM), luminal androgen receptor (LAR), mesenchymal and mesenchymal stem-like (M and MSL) (Lehmann et al., 2011). With all its subtypes and the absence of important receptor markers, the treatment of TNBC is challenging and chemotherapy is believed to be the only efficient treatment (Lehmann et al., 2011). However, in the metastatic phase, chemotherapy also fails and leads to relapse of the tumor and poor patient outcomes (Lin et al., 2008; Wang et al., 2021). Thus, identifying new potential targets is the need of hour to provide an effective antidote in

combination with chemotherapy or surgery. In a recent review, several growth factors and pathways like PI3K/AKT/mTOR, mitogen-activated protein kinase (MAPK), and epithelial-mesenchymal transition (EMT), were summarized that could aid potential new treatment strategies (Lee et al., 2019; Yang et al., 2022). EGFR signaling is one of the key pathways. The EGFR is associated with invasiveness and is expressed at a comparatively higher level in TNBC tumor subtypes than in other subtypes of breast cancer (Hoadley et al., 2007; Nielsen et al., 2004; Viale et al., 2009). Further, the attenuation of EGFR in MDA-MB-231 cells (a human TNBC cell line) leads to a significant decrease in proliferation and migration potential (Manupati et al., 2017).

Simultaneous to the clinical studies where the molecular targets and pathway analysis were performed with patient samples, the *in vitro* studies revealed the involvement of ADAM proteases in TNBC and non-TNBC breast cancer cell lines (Duffy et al., 2009; Lendeckel et al., 2005). ADAMs are also crucially involved in EGFR signaling via EGFR-ligand shedding (Blobel, 2005). Given that ADAM17 is the principle sheddase of EGFR ligands, several studies explored the role of ADAM17 in TNBC in terms of EGFR activation. The increased TGF- α levels were directly reciprocal to ADAM17 levels, and the tumor progression and metastasis were possibly mediated by ADAM17 (McGowan et al., 2007). Furthermore, in MDA-MB-231 cells, the malignant phenotypes such as proliferation, migration, and tube formation were influenced by ADAM17-mediated shedding of TGF- α and activation of the EGFR-PI3K-AKT pathway (Zheng et al., 2009). Further, inhibiting ADAM17 activity led to reduced shedding of TGF- α affecting the migration and invasion of TNBC cells (Giricz et al., 2013). Similarly, selective inhibition of ADAM17 not only reduced cell growth but also decreased the phosphorylation of EGFR to a great extent (McGowan et al., 2013). Moreover, ADAM17 was targeted by specific monoclonal antibodies like D1 (A12) and D8P1C1, resulting in anti-tumorigenic effects (Caiazza et al., 2015; Saha et al., 2022). The anti-tumorigenic properties induced by D8P1C1-mediated inhibition of ADAM17 activity were also confirmed *in vivo* in mouse models (Saha et al., 2022). Amongst other EGFR ligands, soluble HB-EGF and soluble AREG released by ADAM17 expressing cancer cells were reported to induce the release of chemokines from the macrophages that further promote the invasion and tumor progression of the cancer cells (Gnosa et al., 2022). Additionally, ADAM17 has been identified in TNBC cells as a major protease for the shedding of TNFR1 and R2, promoting anti-inflammatory or anti-metastatic effects (Baram et al., 2022).

Besides ADAM17, ADAM8 is highly upregulated in TNBC, and the invasive and metastatic phenotype has been positively correlated with the presence of ADAM8 (Romagnoli et al., 2014). This overexpression of ADAM8 was shown to be a result of hypoxia which results in detachment of tumor cells and transendothelial migration of the tumor. Furthermore, the knockdown of ADAM8 or targeting it with an antibody against its ectodomain alleviates

migration and invasion (Romagnoli et al., 2014). In contrast to ER-positive breast cancer cells (MCF-7), ADAM8 dimers are regularly found on the surface of TNBC cells (MDA-MB-231), suggesting the presence of an active protease (Srinivasan et al., 2014). Upon silencing of ADAM8 in MDA-MB-231 cells, the ability to migrate and penetrate matrigel reduces drastically (Das et al., 2016). The *in vitro* study with MDA-MB-231 cells and an organotypic blood-brain-barrier model showcased the involvement of ADAM8 in the transmigration of breast cancer via the shedding of PSGL-1 and upregulation of MMP-9 (Conrad et al., 2018). Of note, ADAM8 is a known sheddase of PSGL-1 and is also known to regulate its rolling function (Domínguez-Luis et al., 2011). The silencing of ADAM8 by deleting its cytoplasmic domain in MDA-MB-231 cells (MB-231_shA8) leads to reduced levels of MMP9, and its associated activity in progression, which was reversed by re-expression of wildtype ADAM8 (Conrad et al., 2018). Altogether, the tumor microenvironment includes the interaction of different proteins and proteases leading to the activation of signaling pathways controlling invasion and metastasis. Of note, the *in vitro* studies for deletion or knockdown of iRhom1 in breast cancer cells like MDA-MB-231 promoted apoptosis and inhibited invasion and migration (Huang et al., 2018; Zhang et al., 2018).

Considering the aforementioned and the fact that massive ADAM8-dependent degradation of ADAM17 was observed in MDA-MB-231 cells and that some of the identified sADAM17 substrates such as fibronectin, thrombospondin-IV, collagen alpha-2 (IV) and also N-cadherin are known to regulate tumor progression, this raises the possibility of a complex interplay of proteolytic and non-proteolytic functions mediated by ADAM8 and ADAM17 in breast cancer, which requires further investigation.

1.5 Aim of the study

ADAM17 has been described as a canonical ‘full-time sheddase’ whose primary function is the ectodomain shedding of numerous other membrane proteins. With the identification of ADAM8 as a sheddase of ADAM17, it was shown for the first time that this protease can also be proteolytically released, and initial investigations suggested additional so far underestimated roles within the protease web as a soluble enzyme. However, underlying mechanisms regulating the shedding of this essential protease and functional consequences are only poorly understood. Therefore, this thesis aims to contribute to a better understanding of the shedding of ADAM17 to understand in the long term how and where sADAM17 contributes within the protease web to cell homeostasis or pathophysiological conditions.

The first part aimed to further investigate the proteolytic release of ADAM17. Since the ADAM8-mediated shedding of ADAM17 has been studied mainly in an overexpression set up using the murine homologs, the endogenous shedding in the human breast cancer MDA-MB-231 cells should be analyzed in more detail to gain insight into when both proteases start to interact and where in the cell the shedding takes place. Since iRhom1 and 2 are crucial for the regulation of membrane-bound ADAM17, their involvement in ADAM17 shedding should also be investigated.

The second part dealt with the characterization and determination of the proteolytic or shedding function of the soluble ADAM17. Recently an altered substrate spectrum of soluble ADAM17 compared to its membrane-bound counterpart was suggested. However, until now the studies for proteolytic activity were determined using only the recombinant soluble ADAM17. Thus, the overexpression constructs of the active and inactive soluble forms of ADAM17 were generated to explore conclusively distinct and overlapping proteolytic activities of soluble vs membrane-bound ADAM17. Furthermore, to gain some insight into potential events relevant to pathophysiology driven by the ADAM17-ADAM8 interplay in breast cancer a comparative mass spectrometric N-terminomics analysis should reveal novel putative substrates for ADAM8, as well as shared and unique substrates for membrane-bound and soluble ADAM17.

2. Material and Methods

2.1 Chemicals

All chemicals were of analytical grade and obtained from Carl Roth GmbH + Co. KG, Merck KGaA, Sigma-Aldrich Inc., and Thermo Fisher Scientific Inc., if not stated otherwise.

2.2 Buffers and solutions

All generally used buffers and solutions/media are summarized in table 1

Table 1: Overview of buffers and solutions used for the experiments

Buffer	Composition
100% TCA	100% (w/v) trichloroacetic acid
Biotinylation lysis buffer	50 mM TRIS-HCl
	150 mM NaCl
	1% (v/v) Triton X-100
	0.1% (w/v) SDS
	Complete protease inhibition cocktail + EDTA pH 7.4
Blocking buffer for western blot	5% (w/v) milk in 1x TBS or 1x TBS-T
HEPES buffer	20 mM HEPES pH 7.5
	250 mM TRIS-HCl
Laemmli Buffer (5x)	10% (w/v) SDS
	0.5% (w/v) Bromophenol blue
	50% (v/v) Glycerin
	100–200 mM DTT
	pH 6.8
Lysogeny broth Agar	LB medium
	1.5% (w/v) agar-agar
Lysogeny broth media	1% (w/v) tryptone/peptone
	0.5% (w/v) yeast extract
	1% (w/v) NaCl
	135 mM NaCl
PBS	2.7 mM KCl
	9.2 mM Na ₂ HPO ₄
	1.8 mM KH ₂ PO ₄

Materials and Methods

	pH 7.4
PBS-CM	0.1 mM CaCl ₂ 1 mM MgCl ₂ in 1x PBS pH 7.4
Quenching buffer	50 mM Tris-HCl in PBS-CM, pH 8.0
Resolving gel buffer	1.5 M TRIS 0.4% (w/v) SDS pH 8.8
SDS-PAGE buffer (10x)	25 mM Tris-HCl 192 mM Glycine 0.1% (w/v) SDS pH 8.3
SOB media	2% (w/v) tryptone/peptone 0.5% (w/v) yeast extract 0.05% (w/v) NaCl ad 980 ml ddH ₂ O
SOC media	1 ml 2 M glucose (sterile filtered, 0.2 µm) 1 ml 1 M MgCl ₂ (sterile filtered, 0.2 µm) add 100 ml SOB
Stacking gel buffer	0.5 M TRIS 0.4% (w/v) SDS pH 6.8
TAE	40 mM Tris 20 mM acetic acid 1 mM EDTA pH 8.0
Tank blot buffer (10x)	250 mM Tris 2 M Glycine pH 8.3
Tank blot buffer (1x)	100 ml 10x Tank blot buffer 200 ml Methanol 700 ml ddH ₂ O
TBS (5x)	125 mM Tris 750 mM NaCl pH 7.5
TBS-T (5x)	125 mM Tris 750 mM NaCl

0.25% (v/v) Tween20
pH 7.5

2.3 Selection antibiotics and inhibitors

The selection antibiotics for bacterial clones and stably transfected cells and inhibitors are listed in the following table 2.

Table 2: Overview of antibiotics and inhibitors

Inhibitors/ antibiotics and reagents	Composition
1,10- phenanthroline	1M stock in 96% Ethanol
Ampicillin	100 µg/ml in ddH ₂ O
Blasticidin S (Sigma-Aldrich)	10 mg/ml Blasticidin S ready-made solution in 20 mM HEPES buffer (1 mg/ml stocks in 20 mM HEPES buffer, pH 7.5)
Brefeldin A (Biolegend)	1000X solution
EDTA	500 mM in ddH ₂ O
GW280264X (GW, AOBIOUS, Gloucester, MA, USA)	3 mM in DMSO
Kanamycin	100 µg/ml in ddH ₂ O
Penicillin/Streptomycin (Gibco)	10000 U/ml
Puromycin (Merck Millipore)	10 mg/ml stock in ddH ₂ O

2.4 Heat shock transformation and plasmid scale-up

The plasmids were scaled-up via heat shock transformation of *Escherichia coli* (*E. coli*). 1-5 µl (~50-100 ng) template DNA was added to 50 µl freshly thawed chemically competent *E. coli* Top 10 bacteria followed by incubation for 30 min on ice. After a heat shock treatment at 42 °C for 45 s and 2 min incubation on ice, 200 µl super optimal broth with catabolite repression (SOC) medium was added and the reaction mixture was incubated at 37 °C and 300 rpm for 30 mins. Thereafter, 100 µl of the suspension was plated onto lysogeny broth (LB) agar-plates supplemented with ampicillin or kanamycin, and the cultures were incubated at 37 °C overnight. The successfully transformed colonies were picked up to inoculate the mini (3 ml) or midi (100 ml) LB medium culture, supplied with selection antibiotic, and were incubated at 37 °C and 200 rpm overnight. Afterward, plasmid DNA was isolated using the *NucleoBond Xtra Midi kit* (Macherey-Nagel) following the manufacturer's instructions. In the case of mini-

prep, the purified plasmid DNA was eluted with an elution buffer supplied with the kit, and the DNA concentration was determined using the SPARK multimode-microplate reader (Tecan).

2.5 Cloning

2.5.1 Cloning of membrane-bound mADAM17 with HA-tag after pro-domain

The cloning was performed via a two-fragment strategy to generate an overexpressing murine ADAM17 construct ligated in pcDNA 4TO carrying a C-terminal 3xflag-tag. In the membrane-bound murine ADAM17, 2xHA-tag was introduced after the pro-domain, C-terminal to the furin-cleavage site (RVKR) and the construct was named as mADAM17 PD_HA. The primers for cloning (listed in table 3) were designed with the help of a cloning simulation platform, Clone manager 9 (Sci-Ed software, LLC), and were generated by Sigma-Aldrich.

Table 3: List of cloning primers for mADAM17 PD_HA

Template	Cloning Product	Fragment	Primer
mADAM17 WT	mADAM17 PD_HA	F1	F1 mA17 BamHI for 5'-AATCGGATCCATGAGGCGGCGTCT-3'
			mA17 Pro HA R F1 5'-GTCGTAAGGGTACCCGGCGTAGTCGGGCAC GTCGTAAGGGTATCTCTTCACTCGACGAACAAAC-3'
		F2	mA17 Pro HA F F2 5'GCCGGGTACCCTTACGACGTGCCCCGACTACGCCG GCTCCCGAGCTGAACCTAACC-3'
			mA17 Pro HA XhoI R F2 5'-CTACTCGAGGCACTCTGTCTCTTTG-3'

Polymerase chain reaction (PCR) was performed for the two fragments by mixing all the components in the required concentration (shown in table 4). Following the PCR step (table 5), the samples were mixed with 6x Loading Dye (Thermo Fisher Scientific) and loaded on 1% agarose gel prepared by adding 0.6 g of agarose in 60 ml TAE buffer supplemented with 6 µl SYBR™ Safe DNA Gel Stain (10000x, Invitrogen). Gel electrophoresis was run at 100 V for 45 min. Amplified DNA fragments were detected with a UV transilluminator (Intas Science Imaging). The amplified PCR fragments were cut out and purified using the *GeneJET Gel Extraction kit* (Thermo Scientific) following the manufacturer's instructions, and DNA was eluted in 30 µl elution buffer.

Table 4: Components of the PCR reaction mix

Reagents	Final Quantity	Source
Template DNA (100 ng/μl)	0.5 μl	
Forward primer (10 μM)	1 μl	Sigma- Aldrich
Reverse primer (10 μM)	1 μl	Sigma- Aldrich
Deoxynucleoside triphosphate (dNTP) mix (2 mM)	3 μl	Thermo Fisher Scientific
5x Phusion Buffer (GC)	6 μl	Thermo Fisher Scientific
Phusion Polymerase	0.5 μl	Thermo Fisher Scientific
Final volume to 30 μl with DEPC water		

Table 5: PCR program scheme

Step	Time	Temperature	
Initial			
Denaturation	30 s	98 °C	
Denaturation	30 s	98 °C	} 30 cycles
Annealing	30 s	48 °C	
Elongation	120 s	72 °C	
Final elongation	600 s	72 °C	
cooling	∞	4 °C	

The fusion product for mADAM17 PD_HA was generated by fusion PCR protocol adapted from AG Thorsten Mascher (<https://2014.igem.org/Team:LMU-Munich/Notebook/Protocols>). The components were mixed according to table 6 followed by two consecutive programs for the fusion of the fragments (table 7). The gel-purified fused product and the vector were fast digested using *Bam*HI and *Xho*I fast digest enzymes (Thermo Scientific). The digestion was carried out at 37 °C for 5 mins followed by the inactivation at 80 °C for 5 mins as per manufacturer's instructions. The product was again purified by the gel-purification step.

Digested insert and vector were mixed in a 1:5, vector-to-insert ratio in 1x ligase buffer and ligated in the presence of 1 μl T4 ligase (Thermo Scientific). The ligation mix was incubated at 22 °C for 1 h followed by an inactivation at 65 °C for 10 mins. The ligation product was used for heat-shock transformation according to section 2.4. The positive colonies were picked, and plasmid sequences were validated by DNA sequencing (GATC, Eurofins genomics, Germany GmbH).

Table 6: Components of fusion PCR for mADAM17 PD_HA

Reagents	Final Quantity	Source
5x Phusion Buffer (GC)	10 µl	Thermo Fisher Scientific
DEPC	26.8 µl	
Deoxynucleoside triphosphate (dNTP) mix (2 mM)	5 µl	Thermo Fisher Scientific
Phusion Polymerase	0.5 µl	Thermo Fisher Scientific
Fragment 1 (150 ng)	1.73 µl	
Fragment 2 (150 ng)	1.95 µl	
*Forward primer (F1 mA17 BamHI for)	1 µl	Sigma- Aldrich
*Reverse primer (F2 mA17 XhoI rev)	1 µl	Sigma- Aldrich

**to be added after program 1*

Table 7: Fusion PCR program scheme for mADAM17 PD_HA

Step	Time	Temperature	
Program 1			
Initial Denaturation	30 s	98 °C	
Denaturation	10 s	98 °C	} 9 cycles
Annealing	30 s	55 °C	
Elongation	60 s	72 °C	
*Add primers			
Program 2			
Initial Denaturation	30 s	98 °C	
Denaturation	30 s	98 °C	} 30 cycles
Annealing	30 s	55 °C	
Elongation	150 s	72 °C	
Final elongation	600 s	72 °C	
cooling	∞	4 °C	

2.5.2 Cloning of ectodomain of murine ADAM17

The ectodomain of murine ADAM17 was ligated into pcDNA 4TO carrying c-terminal 3xflag-tag. The construct was generated by introducing an N-terminal 2xHA tag between the signal and the pro-peptide, and the construct was named HA_sADAM17_flag. The primers and sequence are mentioned in table 8.

Table 8: List of cloning primers for HA_sADAM17_flag

Template	Cloning Product	Fragment	Primer
mADAM17 WT	HA_sADAM17 _flag	F1	F1 mA17 BamHI for 5'-AATCGGATCCATGAGGCGGCGTCT-3'
			F1 mA17 HA rev 5'GTCGTAAGGGTACCCGGCGTAGTCGGGCACGTCG TAAGGGTATGCCAGGACGAAAG-3'
		F2	F2 mA17 HA for 5'- GCCGGGTACCCTTACGACGTGCCCCGACTACGCCGG C TCCCCCGACCT-3'
			F2 mA17 XhoI rev 5'-GATTCTCGAGGTTATCTGCCAGAACTTCC-3'

For the PCR amplification, the components were mixed as shown in table 4. The annealing temperature for fragment 1 (F1) was 48 °C and for fragment 2 (F2) 49 °C, and the PCR program was the same as shown in table 5. The fusion product for the HA_sADAM17_flag construct was generated by fusion PCR and the PCR program was the same as shown in table 5. The purified fragments were mixed according to table 9 and annealed at 53 °C. The fused product was then once again purified via gel purification.

Table 9: Components of fusion PCR for sHA_mADAM17_flag

Reagents	Final Quantity	Source
5x Phusion Buffer (GC)	6 µl	Thermo Fisher Scientific
Deoxynucleoside triphosphate (dNTP) mix (2 mM)	3 µl	Thermo Fisher Scientific
Forward primer (F1 mA17 BamHI for)	1 µl	Sigma- Aldrich
Reverse primer (F2 mA17 XhoI rev)	1 µl	Sigma- Aldrich
Fragment 1	9 µl	
Fragment 2	9 µl	
Phusion Polymerase	0.5 µl	Thermo Fisher Scientific
Final volume to 30 µl with DEPC water		

The gel-purified fusion product and the vector were digested using *BamHI* and *XhoI* restriction digestion enzymes (Thermo Scientific). The digestion was carried out at 37 °C for 2 h followed by enzyme inactivation at 80 °C for 20 mins according to the manufacturer's instructions.

Purified and digested insert and vector were mixed in a 1:3 vector to insert ratio in 1x ligase buffer and ligated in the presence of 1 µl T4 ligase (Thermo Scientific). The ligation mix was incubated at 37 °C for 1 h followed by an inactivation at 70 °C for 10 mins. The ligation product was used for heat-shock transformation and positive colonies were picked, plasmid DNA isolated, and sequences validated by DNA sequencing (GATC, Eurofins genomics, Germany GmbH).

2.6 Cell culture

WT HEK 293T (DSMZ GmbH, Germany), ADAM10/17^{-/-} HEK293T (kindly provided by Dr. Björn Rabe, University of Kiel, Germany (Riethmueller et al., 2016) were maintained in Dulbecco's modified eagle medium with GlutaMAX™ (DMEM; Gibco) supplemented with 10% (v/v) Fetal calf serum (FCS; Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin under humidified conditions (5% CO₂) at 37 °C. WT mEF and iRhom1/2^{-/-} mEF (kindly provided by Dr. Colin Adrain; (Christova et al., 2013) were also maintained in DMEM with GlutaMAX™ supplemented with 10% (v/v) FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin under humidified conditions (5% CO₂) at 37 °C. The stably transfected mEF cells with iR2_HA and the mutants iR2_dead_HA and iR2_dead R18_HA were maintained in growth media supplied with an additional 5 µg/ml Blasticidin S (Sigma-Aldrich). Mammalian triple-negative breast cancer cells (MDA-MB-231 variants) were kindly provided by Prof. Dr. J. W. Bartsch. WT MDA-MB-231 cells were maintained in DMEM without sodium pyruvate (DMEM; Sigma-Aldrich), supplied with 10% (v/v) FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin and MDA-MB-231 hADAM8 KO (hA8 KO) and hADAM8 E335Q (hA8 E>Q) mutants were maintained with additional 2 µg/ml puromycin (Merck Millipore) under humidified conditions (5% CO₂) at 37 °C.

2.7 Transient transfection

For the transient transfection, all the plasmids are listed in table 10. Variants of HEK 293T cells were seeded at a density of 2.5-3x10⁶ cells per 10 cm dish and transfected upon reaching 80-90% confluency. For each 10 cm dish, a final concentration of 6 µg plasmid DNA was mixed with Polyethyleneimine (PEI, Polysciences) transfecting reagent. For every 1 µg plasmid DNA, 3 µl PEI (1 mg/ml in ddH₂O) was mixed in serum-free media followed by 30 mins incubation at room temperature. After the transfection, cells were incubated at 37 °C, 5% CO₂ for 5 h, and fresh serum-free media was supplied to the dishes. The cells were incubated at 37 °C, 5% CO₂ over the night. Afterward, cells were washed 2 times with 1x Dulbecco's phosphate buffer saline (DPBS; gibco), and 5 ml fresh serum-free medium was added to the 10 cm dishes. Depending upon the experiment, the cells were incubated at 37 °C, 5% CO₂ for 6-24 h. For

experiments with conditioned media analysis for shedding of the substrates, the conditioned media was collected from the dishes after 6 h starvation and transferred to dishes singly transfected with substrate plasmids. The cells were further incubated for 6 h under humidified conditions.

mEF cells were seeded in T175 flasks at a density of $2.5\text{--}3 \times 10^6$ cells. The cells were transfected with 9 μg of plasmid in the presence of PEI as mentioned above. The cells in T175 flasks were starved in serum-free media and incubated for 24 h. MDA-MB-231 cells were seeded at a density of 3×10^6 cells per 10 cm dish and transfected with 3 μg of hADAM17 DNA with PEI as a transfection reagent as described above. The cells were starved in DMEM only without sodium pyruvate for 24 h before harvest. For the inhibition study against ADAM17 shedding, the shedding of endogenous and overexpressing ADAM17 was analyzed in 10 cm dishes where after reaching 80-90% confluency, cells were washed twice with 1xDPBS and supplied with fresh DMEM without sodium pyruvate and supplied with 500 μM EDTA, 100 μM 1,10-phenanthroline, 3 μM GW, 5 $\mu\text{g}/\text{ml}$ Brefeldin A and DMSO and incubated at 37 °C, 5% CO₂ for 12-24 h before harvest.

Table 10: Overview of plasmids used for transient transfection

Plasmid name	Tag	Vector	Species	Antibiotic resistance
pcDNA 3.1/ Empty vector	-	pcDNA 3.1	-	Ampicillin
hADAM17	C_myc_flag	pCMV6- entry	human	Kanamycin
mADAM17 E406A (mADAM17 E>A)	-	pcDNA 3.1	murine	Ampicillin
mADAM17 PD_HA	HA tag after pro- domain, C_flag	pcDNA 4TO	murine	Ampicillin
mADAM17 WT	-	pcDNA 3.1	murine	Ampicillin
HA_sADAM17_flag	N_HA, C_flag	pcDNA 4TO	murine	Ampicillin
HA_sADAM17_flag E406A (HA_sADAM17_flag E>A)	N_HA, C_flag	pcDNA 4TO	murine	Ampicillin
Amphiregulin (AREG.AP)	N_AP	pAIPh	human	Ampicillin
Betacellulin (BTC-AP)	N_AP	pAIPh	human	Ampicillin
Epiregulin (EREG-AP)	N_AP	pAIPh	human	Ampicillin
Heparin-binding-EGF (HB-EGF-AP)	N_AP	pAIPh	human	Ampicillin
Tumor growth factor α	N_AP	pAIPh	human	Ampicillin

(TGF- α -AP)

The inactive variants of membrane-bound murine ADAM17 (mADAM17 E>A) and the ectodomain of ADAM17 (HA_sADAM17_flag E>A) were generated by site-directed mutagenesis (GAA>GCC) by Eurofins genomics, Germany GmbH.

EGFR-AP ligand plasmids were kindly provided by Dr. Carl. P. Blobel (Sahin et al., 2004).

2.8 Retroviral transduction

The retroviral transduction method was adapted and modified from two previously published protocols for iRhom2 stable transfection (Schwarz et al., 2013; Siggs et al., 2014). 3×10^6 WT HEK 293T cells were seeded per 10 cm dish, after reaching an 80% confluency, cells were transfected with 3 μ g of viral packaging plasmid (pCL-ECO) and 3 μ g of target expression plasmid (pM6P.blast) containing cDNA for C-terminally HA-tagged, wild-type or mutant iRhom2 (iR2_HA, iR2 dead_HA or iR2 dead R18_HA). The packaging vector and the mutant plasmids were kindly provided by Dr. Colin Adrain (Cavadas et al., 2017). The transfection was carried out with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. For 1 μ g plasmid DNA mixed with 2 μ l P3000, 3 μ l Lipofectamine 3000 was supplied in serum-free media. The transfection mix was supplied to the cells after 15 mins incubation at room temperature followed by an overnight incubation at 37 °C, 5% CO₂. After the incubation, the cells were washed 2 times with 1x DPBS and 5 ml fresh media with 10% FCS was supplied for the secretion of the virus for 20 h. The supernatant was collected and centrifuged at 1000x g for 5 mins at room temperature and subsequently filtered with a 0.45 μ m syringe filter. The virus-rich media was then diluted 1:2 and 2 ml of diluted media was supplied to the iRhom1/2^{-/-} mEF cells seeded in a 6-well plate in the presence of a 50 μ g/ml polybrene infection reagent. To sediment the viral particles, the plates were centrifuged at 1000x g for 2 h, room temperature, and incubated at 37 °C, 5% CO₂ for 24 h. Thereafter, the wells were washed once with 1xDPBS and 3 ml fresh media with 10% FCS was supplied, followed by 48 h incubation, and stable clones were selected with 5 μ g/ml Blasticidin S for 14 days with occasional splitting.

2.9 Cell lysis and TCA precipitation

Cells were harvested after the defined incubation time with the help of a cell scraper in ice-cold 1x DPBS and centrifuged at 3000 rpm for 3 mins. The cell pellets were washed 2 times with 1x DPBS and resuspended in 200 μ l lysis buffer with EDTA [Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland), 1% Triton X-100, PBS, pH 7.4] and incubated on the roller at 4 °C for 2h. Cell lysates were centrifuged at 15000x g, 4 °C for 15 mins. The protein

concentration was determined by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) following the kit manual.

The supernatant collected during the cell harvest was centrifuged at 3000 rpm, 4 °C for 5 mins to get rid of cell debris and called as cleared supernatant. When required, supernatant from each sample was ultracentrifuged at 186000x g, 4 °C for 2 h to get rid of microvesicles. Ultracentrifuged or cleared supernatant was TCA (tri-chloro-acetic acid) precipitated by adding 10% (v/v) TCA solution (stored at room temperature). After 60 mins of incubation on ice, the tubes were centrifuged at 15000x g, 4 °C for 15 mins. The supernatant was discarded, then 200 µl chilled acetone was added to the pellet and the tubes were shortly vortexed. The samples were then centrifuged at 15000x g, 4 °C for 8 mins, and the pellets were re-solubilized in 40-50 µl pre-warmed 1x Laemmli buffer with DTT, followed by incubation at 95 °C for 10 mins.

2.10 SDS-PAGE and Western blot

The proteins in cell lysates and supernatant were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), using the Mini-PROTEAN® system (Bio-Rad). The stacking-gel concentration was 4% and resolving-gel was cast at a concentration of 7.5 - 12% as per requirement (composition shown in table11). For the lysates, if not stated otherwise, 30 µg of total protein and for the TCA precipitated supernatants, volumes normalized to lysate protein concentrations were loaded onto the gels. The gels were run at 125 V for 90 mins and proteins were transferred onto nitrocellulose membrane or PVDF membrane via tank blot at 800 mA, 4 °C for 2 h. Membranes were further blocked with 5% milk/TBS or 5% milk/TBS-T at room temperature for 1 h followed by overnight incubation at 4 °C with primary antibodies (Table 12). Thereafter, the membranes were incubated with HRP-conjugated secondary antibodies diluted 1:10000 in 5% milk/TBS or TBS-T at room temperature for 1h (Table 13). The chemiluminescence was detected using either the SuperSignal West Femto or Pico Kit (Thermo Fisher Scientific) or ECL substrate kit (Advansta, K-12045-D20) according to the manufacturer's instructions, and the blots were developed using the LAS-3000 Imaging System (FUJIFILM Europe GmbH) or Imagequant 800 (Amersham).

Table 11: Resolving and stacking gel recipe for SDS-PAGE analysis

Components	1X Resolving gel			1X Stacking gel
	7.5%	10%	12%	4%
Resolving gel buffer	2.00 ml	2.00 ml	2.00 ml	
Stacking gel buffer				1.00 ml
ddH ₂ O	3.88 ml	3.21 ml	2.68 ml	2.44 ml
30% (37.5:1) Acrylamide	2.00 ml	2.67 ml	3.20 ml	510 µl
10% (v/v) TEMED	80 µl	80 µl	80 µl	50 µl
10% (w/v) Ammonium persulfate	80 µl	80 µl	80 µl	50 µl

Table 12: List of primary antibodies used in western blot analysis

Antibody	Species	dilution	Source
hADAM17 [Ab19027]	rabbit	1:500 in 5% milk/TBS	Merck-Millipore
hADAM17 [A300D]	mouse	1:500 in TBS-T	Institute of Biochemistry, CAU-Kiel (Trad et al., 2011)
mADAM17 [10.3]	rabbit	1:1000 in 5% milk/TBS	Pineda Antikörper-Service, Berlin, Germany
GAPDH [14C10]	rabbit	1:1000 in 5% milk/TBS	Cell Signaling
HA [C29F4]	rabbit	1:1000 in 5% milk/TBS	Cell Signaling
Flag [M2]	mouse	1:1000 in 5% milk/TBS	Sigma-Aldrich
Alkaline-phosphatase [ab133602]	rabbit	1:1000 in 5% milk/TBS	Abcam
hADAM8 [PA5-47047]	goat	1:500 in 5% milk/TBS-T	Thermo-Fischer scientific
Myc [9B11]	mouse	1:1000 in 5% milk/TBS	Cell Signaling
Pan-cadherin [4068]	rabbit	1:1000 in 5% milk/TBS	Cell Signaling
Transferrin- receptor [ab84036]	rabbit	1:1000 in 5% milk/TBS-T	Abcam
iRhom1	rat	1:10 in TBS-T	AG-Lichtenthaler (Weskamp et al., 2020)
iRhom2	rat	1:10 in TBS-T	AG-Lichtenthaler (Weskamp et al., 2020)

Table 13: List of secondary antibodies used in western blot analysis

Antibody	Species	dilution	Source
Anti-rabbit-IgG (H+L)-HRP	goat	1:10000 in 5% milk/TBS	Jackson ImmunoResearch
Anti-mouse-IgG (H+L)-HRP	sheep	1:10000 in 5% milk/TBS	Jackson ImmunoResearch
Anti-goat-IgG (H+L)-HRP	donkey	1:10000 in 5% milk/TBS	Jackson ImmunoResearch
Anti-rat-IgG (H+L)-HRP	goat	1:10000 in 5% milk/TBS	Jackson ImmunoResearch

2.11 Immunofluorescence

For the colocalization analysis of proteins, MDA-MB-231 cells were seeded at a density of 1.5×10^5 cells per well in a 6 well-plate on 13 mm glass coverslips followed by an incubation of 24-48 h until the cells were 70-80 % confluent. The cells were washed 3 times with 1x DPBS and fixed with 4% (w/v) PFA in PBS for 10 mins at room temperature. When required, the cells were then permeabilized with 0.2 % saponin and 0.12 % glycine in 1x PBS for 10 mins at room temperature. The blocking was done for 1 h at room temperature with a buffer containing 10% FCS and 0.2% saponin in PBS. The primary antibodies (listed in table 14) were diluted in 10% FCS in PBS and incubated at 4 °C, overnight. The coverslips were washed 5 times with 0.2 % saponin in PBS and incubated for 1 h with Alexa-fluor conjugated secondary antibodies (listed in table 15) diluted 1:300 and 1 µg/ml DAPI (Roth). The coverslips were then washed 5 times with 0.2 % saponin in PBS and twice with ddH₂O and finally mounted on glass slides with 15µl mounting medium DAKO (S3023, Agilent, Santa Clara, CA, USA). Immunofluorescence imaging was performed using the confocal microscope Zeiss LSM980 microscope with 63x objective (c-plan apochromat 63x /1.4 oil DIC) (Carl Zeiss Microscopy GmbH, Jena, Germany). The images were processed using Zen 3.1 blue edition software (Carl Zeiss Microscopy GmbH).

Table 14: List of primary antibodies used in immunofluorescence microscopy

Antibody	Species	dilution	Source
hADAM17 [Ab19027]	rabbit	1:500	Merck-Millipore
hADAM8 [PA5-47047]	goat	1:40	Thermo Fischer scientific
ERp5 [F5]	mouse	1:250	Cell Signaling
EGFR [66455-1-Ig]	mouse	1:500	Proteintech

Table 15: List of secondary antibodies used in immunofluorescence microscopy

Antibody	Species	dilution	Source
AlexaFluor-488 IgG (H+L)	donkey	1:300	Invitrogen
AlexaFluor-594 IgG (H+L)	donkey	1:300	Invitrogen
AlexaFluor-647 IgG (H+L)	donkey	1:300	Invitrogen

2.12 Glycoprotein enrichment and deglycosylation

ADAM10/17^{-/-} and iRhom1/2^{-/-} HEK 293T cells were transfected with mADAM17 WT and HA_sADAM17_flag as stated in section 2.7. The supernatant was collected, and cells were harvested and lysed for 1 hour at 4 °C in buffer with EDTA [Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland), 1 % Triton X-100, 150 mM NaCl, 50 mM Tris, 10 mM 1,10-phenanthroline, PBS, pH 7.4]. Then, glycosylated proteins in the lysates and the supernatant samples were enriched by concanavalin A beads. Lysates were cleared by centrifugation at 15000x g, 4 °C for 15 mins; consequently, 50 µl concanavalin A beads (Sigma Aldrich) were added to two tubes each with 450 µl lysate containing 100 µg of protein and incubated at 4 °C for 3 h with overhead rotation. The beads were washed, and the proteins were finally eluted. From two tubes, one tube was untreated, and the other was taken for treatment with PNGase F or Endo H. Proteins were eluted in 1x Laemmli buffer supplemented with DTT for the untreated sample. The proteins in the second tube were eluted with 1x glycoprotein denaturing buffer (NEB, Ipswich, MA, USA) for 10 mins at 95 °C.

For the enrichment of glycosylated proteins in the supernatant, supernatant from 3x 10cm dishes were combined from each cell line setup. Circa 12.5 ml supernatant was concentrated down to 3 ml using Amicon Ultra-15 centrifugal filter units (10 kDa cutoff, Merck Millipore Ltd.). 40 µl of 50x Protease inhibitor solution was added to avoid any degradation. The concentrated supernatant was then ultracentrifuged at 186000x g, 4 °C for 2 h followed by the addition of 100 µl concanavalin A beads washed in PBS to 900µl of supernatant (volume adjusted according to lysate protein concentration) and overnight incubation at 4 °C. The beads were washed, and the proteins were finally eluted in 50 µl 2x Laemmli buffer or 1x glycoprotein denaturing buffer (NEB, Ipswich, MA, USA) for 10 mins at 95 °C. After the enrichment, 20 µg of glycoproteins were deglycosylated by applying 1 unit of Endo H and PNGase F according to the manufacturer's instructions (NEB, Ipswich, MA, USA).

2.13 AP-activity assay

For the shedding activity analysis, an alkaline phosphatase (AP) activity assay was performed with cell supernatants of the transfected HEK 293T cells. Supernatants from each sample were

ultracentrifuged at 186000x g, 4 °C for 2 h to get rid of microvesicles, for the analysis of shedding of various EGFR-AP ligands (Tokumaru et al., 2000), the volume of supernatants was adjusted according to the protein concentration of the respective lysates. After volume adjustment, 100 µl of the sample was added to a 96-well plate in duplicate, and 100 µl substrate solution was supplied to each well [2mg/ml para- nitrophenyl-phosphate (4-NPP), (Sigma)] in alkaline-phosphatase assay buffer] (100 mM Tris, 100 mM NaCl, 20 mM MgCl₂ in ddH₂O, pH 9.5). The activity was measured continuously for 7 h in 15-60 mins cycles at 405 nm.

2.14 Cell surface biotinylation

To label cell surface proteins with biotin in ADAM10/17^{-/-} HEK 293T cells, cells were transfected in 10 cm dishes according to section 2.7. The cells were starved in serum-free DMEM for 24 h. For biotinylation in iRhom1/2^{-/-} mEF cells, transfection was done with 9 µg mADAM17 variants, and cells were cultured in T175 flasks. Similarly, for biotinylation of endogenous cell surface proteins in MDA-MB-231 cells, cells were cultivated in T175 flasks and starved in serum-free media for 24 h. After incubation, cells were cooled down at 4 °C for 10 mins followed by a washing step with PBS-CM. 3 ml of Sulfo-NHS-SS-Biotin (1 mg/ml in PBS-CM; Thermo Fisher Scientific) were applied to 10 cm dishes and 9 ml to T175 flasks for 30 mins at 4° C. Afterwards, the biotin solution was removed, and 10 ml quenching buffer was added for 10 min at 4 °C. After washing the cells 3 times with PBS-CM, cells were scrapped and lysed in 200 µl biotinylation lysis buffer for 30 mins at 4 °C. The harvested cells were then centrifuged at 16000x g, 4 °C for 15 mins. 50 µl of the lysate was used for protein concentration determination using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) and as lysate control (30 µg) in Western blot analysis. The remaining lysate was incubated with 50 µl of Pierce™ Streptavidin Magnetic Beads (Thermo Fisher Scientific). Then, a total of 350 µg of biotinylated protein in the case of HEK 293T and MDA-MB-231 cells and 600 µg of biotinylated proteins in the case of mEF cells were mixed with 50 µl beads in a total volume of 500 µl and incubated at 4 °C for 90 min on rotation. The samples were placed on a magnetic stand to remove the supernatant and the beads were washed 3 times with 500 µl TBS-T. Samples were eluted by incubating the beads in 100 µl 1x Laemmli buffer at 95 °C for 5 mins for SDS-PAGE and Western blot.

2.15 Fluorogenic peptide-based activity assay

A fluorogenic peptide-based activity assay was performed to investigate the proteolytic activity of the overexpressing sADAM17 (HA_sADAM17_flag) and endogenously released sADAM17 in the conditioned media. The activity was measured using the substrate Dabcyl-

PRAAAHomopheTSPK(5FAM)-NH₂ (PEPDAB064, BioZyme Inc., Apex, NC, USA) and TNF- α based peptide; Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH₂; (ES003, R&D systems). For activity determination of sADAM17, the volume of ultracentrifuged or cleared supernatants was adjusted according to protein concentration in the respective lysates. The supernatants were then supplied with 3 μ M GW or with 1:100 diluted DMSO in 96-well plates. After 30 mins incubation at room temperature, 10 μ M fluorogenic substrate was added and fluorescence was measured at λ_{em} =530 nm and λ_{ex} =485 nm for PEPDAB064 and λ_{em} =320 nm and λ_{ex} =405 nm for TNF- α based peptide at 37 °C every 30 s throughout 2 h with a spectrophotometer (Infinite F200Pro, Tecan, Mannedorf, Switzerland). For the bar graph presentation of data, the relative fluorescence units (RFU) were used. The increase in RFU was normalized to the initial point of measurement and the data were analyzed by GraphPad Prism (La Jolla, CA, USA).

2.16 N-terminomics for new substrate identification

2.16.1 Sample preparation for TAILS analysis

For the identification of new substrates, the cell supernatant from MDA-MB-231 cells was subjected to N-terminomics analysis. For this, WT, hA8 KO, and hA8 E>Q MDA-MB-231 cells were grown to ~80% confluency in T175 flasks. Cells were then washed 3 times with 1x DPBS (Gibco) and grown in DMEM medium (Sigma Aldrich) without serum and phenol red and incubated under humidified conditions at 37 °C, 5% CO₂ for 24 h. Thereafter, a second media change with fresh serum-free and phenol red-free medium was given, and cells were incubated at 37 °C, 5% CO₂ for 24 h. For the conditioned media setup, the serum-free medium was either supplied with or without 3 μ M GW (a potent ADAM17 inhibitor) to WT cells. Following incubation, the secretome was collected, and 1x protease inhibitor cocktail (cOmplete™, Roche Deutschland Holding GmbH) was immediately added to avoid any proteolytic degradation. A flow scheme for the sample preparation till the supernatant harvest is shown in Figures 7A and 7B.

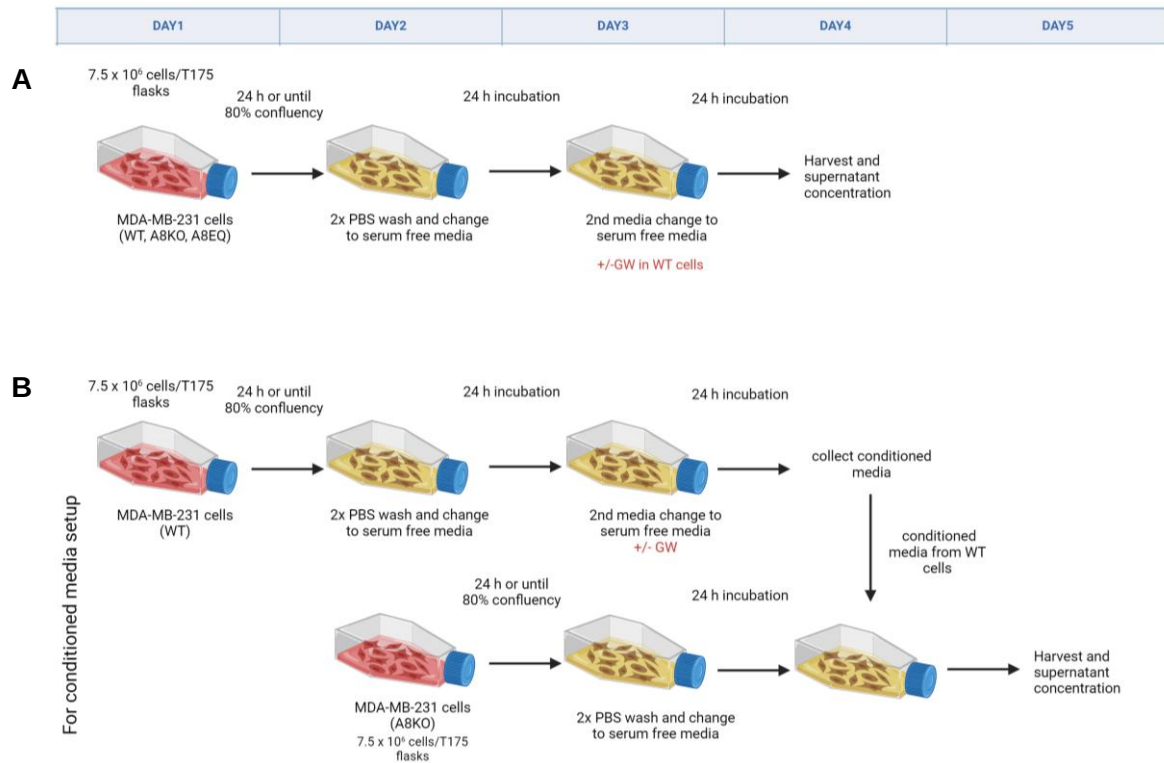


Figure 7: Scheme for sample preparation for N-terminomics analysis

(A) WT, hA8KO, and hA8E>Q MDA-MB-231 cells were seeded at a density of 7.5×10^6 cells per T175 flasks and were grown to 80% confluency. The cells were washed twice with 1x DPBS and starved in serum-free media for 24 h. Fresh serum-free media change was given once again followed by 24 h incubation. In the case of WT cells, the media was supplied with or without $3 \mu\text{M}$ GW. The secretomes were harvested for further steps after 24 h incubation.

(B) For the conditioned media setup, WT MDA-MB-231 cells were seeded at a density of 7.5×10^6 per T175 flasks and were grown to 80% confluency. 24 h later, the cells were washed twice with 1xDPBS and starved in serum-free media. After 24 h, medium exchange with fresh serum-free media was supplied with or without $3 \mu\text{M}$ GW. After the incubation, the conditioned media was collected and transferred to hA8 KO cells followed by incubation of 24 h and collection of secretome for further analysis.

From the harvested supernatant samples, the cell debris was removed by centrifugation at $2200 \times g$, 4°C for 5 mins, and sterile filtration ($0.22 \mu\text{m}$). The conditioned media were further concentrated at 4°C by ultrafiltration using Amicon Ultra-15 centrifugal filter units (10 kDa cutoff, Merck Millipore Ltd.). Using the same Amicon concentrators, the buffer exchange to 100 mM HEPES (pH 7.0) by five cycles of dilution and concentration was carried out. Protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The samples were then aliquoted at a concentration of 1-2 mg/ml, snap-frozen in liquid nitrogen, and stored at -80°C until further use. Additionally, for the identification of specific sADAM17 substrates, hA8 KO cells were supplied with the conditioned media (with or without GW) collected from the WT cells. hA8 KO cells were further incubated at 37°C , 5% CO_2 for 24 h, followed by the concentration of the collected supernatant and buffer exchange.

2.16.2 TAILS

The N-terminomic TAILS analysis was done in collaboration with Prof. Dr. Ulrich auf dem Keller, Department of Biotechnology and Biomedicine, DTU, Lyngby, Denmark. All individual experiments were performed from secretome aliquots of the same batch and in three technical replicates each. For the TAILS analysis, 50 µg of protein for each condition was denatured in 2.5 M GuHCl, 250 mM HEPES, pH 8.0 for 15 mins at 65 °C. Cysteine residues were reduced by the addition of tris (2-carboxyethyl) phosphine (TCEP) to a final concentration of 5 mM and incubated at 65°C for 45 mins, followed by alkylation in the presence of 5 mM chloroacetamide (CAA) at 65 °C for 30 mins. The proteins in each sample were labeled at a 1:4 protein: TMT (w/w) ratio with TMT reagents (TMT18plex labeling Kit; Thermo Fisher Scientific, Waltham, MA) for 1h at room temperature. The reaction was quenched with 100 mM ammonium bicarbonate at RT for 30 mins. The labeled samples were combined and precipitated by adding eight sample volumes of ice-cold acetone and one sample volume of ice-cold methanol at – 80°C for 2 h. After centrifugation at 4700x g at 4 °C for 30 min, a wash step with 5 ml ice-cold methanol, and another round of centrifugation the pellet was air-dried. The pellet was resuspended in 100 mM NaOH and adjusted with 1 M HEPES to 1 µg/µl protein in 100 mM HEPES, pH 7.8. The protein sample digestion was carried out using trypsin (Trypsin Gold, Promega) at 1:50 enzyme: protein ratio (w/w) for 16h at 37 °C. The following steps for the enrichment of N-terminal blocked peptides were carried out according to Madzharova et al. (Madzharova et al., 2019). For desalting C18 EvoTips (EvoSep) were activated by adding 100 µl isopropanol for 1 minute. After addition of 50 µL buffer B (80% Acetonitrile, 0.1% Formic acid), followed by centrifugation at 1400x g, the tips were then re-soaked in isopropanol for 1 minute and centrifuged again at 1400x g for 10 s. Then buffer A (0.1% Formic acid) was added to each EvoTip followed by centrifugation at 700x g for 1 min. The sample (500 ng) in buffer A was loaded onto the EvoTip, followed by centrifugation at 1400x g for 30 s, and two centrifugation steps after adding 50 µl buffer A. Lastly, 250 µl buffer A was added to each EvoTip and spun at 300x g for 10 s. The EvoTip trap columns were subsequently transferred to the EvoSep One liquid chromatography system and scheduled for analysis with LC-MS/MS.

2.16.3 LC-MS/MS analysis

For LC-MS/MS analysis, an EvoSep One platform (EvoSep One) was used in-line with an Exploris 480 Orbitrap mass spectrometer (Thermo Scientific), equipped with a FAIMSpro ion mobility device. Peptides were loaded on an EV1106 C18 column (15cm x 150 µm, 1.9 µm diameter) and separated using the 10 samples per day (10SPD), 118 min gradient with Whisper100 (100 nl/min) nanoflow. Eluting peptides were injected into the mass spectrometer, operated in data-dependent mode, using a 20 µm fused silica emitter (EV1087) with nano-

spray ionization, a spray voltage of 2300 V, carrier gas flow of 3.6 L/min, and ion transfer tube temperature of 204 °C. Three different compensation voltages of -40, -55, and -70 were applied for FAIMS ion filtering during data acquisition. For each compensation voltage cycle, the top 10 precursors in the MS scan were selected for MS/MS sequencing. For all cycles, MS1 scans were acquired with an Orbitrap resolution of 120,000, a scan range of 375-1500 m/z, and RF Lens focusing at 60%. Maximum injection time was set to Auto, and the normalized AGC target was set at 300%. Before MS/MS sequencing, the following filters were applied: MIPS filter on peptide mode, intensity threshold of 5000, a charge state range of 2-7, dynamic exclusion set to 60 s duration and mass tolerance at 10 ppm, and precursor fit with a threshold of 70%. MS/MS scans were acquired in centroid mode at 60,000 resolution after precursor isolation with a 0.7 m/z isolation window and fragmentation with 34% NCE. Maximum injection time set to auto, with a normalized AGC target of 75% and a first mass of 110.

2.16.4 Data analysis and normalization

The acquired raw data were analyzed with the Proteome Discoverer software, version 2.4. Peptide spectrum matches (PSMs) were generated with the SequestHT engine, and FDR was controlled with Percolator. Spectra were matched against the human proteome (reviewed, 40,297 sequences, accessed 03/30/2021) using modified semi-tryptic specificity (trypsin_R, like ArgC, with open N- or C-terminal terminal cleavage) allowing for 1 missed cleavage, with a minimum and maximum peptide length of 6-36 residues, respectively. Precursor tolerance was set to 10 ppm and fragment mass tolerance to 0.02 Da. The dynamic modifications of methionine oxidation (+15.995 Da), asparagine deamidation (+0.984) and N-terminal acetylation (+42.011 Da) or TMTpro N-terminal modification (+304.207 Da) were added to the search, as well as static modifications for cysteine carbamidomethylation (+57.021 Da) and lysine TMTpro modification (+304.207). The percolator was set to 1% in strict mode and 5% in relaxed mode. Peptide and protein quantification were normalized based on the total peptide amount in each channel, excluding TMTpro N-terminal modified peptides. Unique peptides were considered for protein quantification. Similarly, PSMs with a co-isolation threshold higher than 50% and average signal-to-noise ratios below 10 were filtered and not considered for peptide quantification. No scaling was applied, while differential abundance was determined with protein-abundance-based protein ratio calculation and hypothesis testing was performed with ANOVA on individual proteins.

2.17 Statistical analysis and illustrations

All the graphs were plotted with GraphPad Prism 8.0 software. The statistical analyses were performed with the GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA). For more than two data sets, a one-way analysis of variance (ANOVA) with Dunnett's post-hoc test was applied (ns: $p > 0.05$; *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$). The heat maps and volcano plots for N-terminomics TAILS data were also generated by GraphPad Prism 8.0. N-termini were considered significant if they had a p-value less than or equal to 0.01, and a log 2-fold change of ± 1.585 or ± 0.585 in the case of conditioned media samples. All figures were created with Microsoft PowerPoint and BioRender.com.

3. Results

3.1 ADAM8 mediated shedding of ADAM17 in breast cancer cells

In human triple-negative breast-cancer cells (MDA-MB-231), metastasis, invasion, angiogenesis, and proliferation were positively associated with the high expression of ADAM8 (Conrad et al., 2018; Cook et al., 2022; Romagnoli et al., 2014) and ADAM17 (McGowan et al., 2013; Zheng et al., 2009). The involvement of ADAM8 and ADAM17 in the same cellular events implies a correlation between the proteolytic and non-proteolytic activities of both proteases. As mentioned earlier, the *in vitro* studies performed in MDA-MB-231 cells showed a decrease in the levels of sADAM17 upon knockout of ADAM8 (Scharfenberg et al., 2020). Additionally, through a co-immunoprecipitation experiment, a direct protein-protein interaction between ADAM8 and ADAM17 was also reported (Scharfenberg et al., 2020). Therefore, to further elucidate the interaction between ADAM8 and ADAM17, MDA-MB-231 cells that endogenously express both proteins, were analyzed by applying immunofluorescence imaging and cell surface biotinylation of the proteins.

3.1.1 Interaction of ADAM17 and ADAM8 on the cell surface

In addition to the elevated expression of ADAM8 and ADAM17 in MDA-MB-231 cells, high cell surface expression of the EGFR was also detected in these cells (Cahall et al., 2015; Fitzpatrick et al., 1984; Manupati et al., 2017). Additionally, since the involvement of ADAM8 and ADAM17 in EGFR signaling is well known, EGFR was selected to confirm the colocalization of ADAM8 and ADAM17 on the cell surface. The colocalization was analyzed by immunofluorescence microscopy carried out on MDA-MB-231 wild-type (WT) cells, ADAM8 knockout (hA8 KO), and cells expressing the catalytically inactive ADAM8 E335Q mutant (hA8 E>Q). The cells were grown on coverslips to 70-80% confluency and were permeabilized with 0.2% saponin after fixation.

ADAM17 was stained with an antibody against the C-terminus of ADAM17 (Ab19027, 1:500 in 10% FCS in PBS), ADAM8 was stained with an antibody raised against its ectodomain (PA5-47047, 5 µg/mL in 10% FCS in PBS), and EGFR was stained with anti-EGFR antibody specific to ectodomain (66455-1-Ig, 1:250 in 10% FCS in PBS). ADAM17 (green) appeared to be highly localized with EGFR (orange) on the cell membrane in WT (Figure 8A), hA8 KO cells (Figure 8B), and hA8 E>Q cells (Figure 8C). On the other hand, ADAM8 (red), appeared to be slightly less prominent on the cell membrane in hA8 E>Q cells (Figure 8C) as compared to the WT cells (Figure 8A).

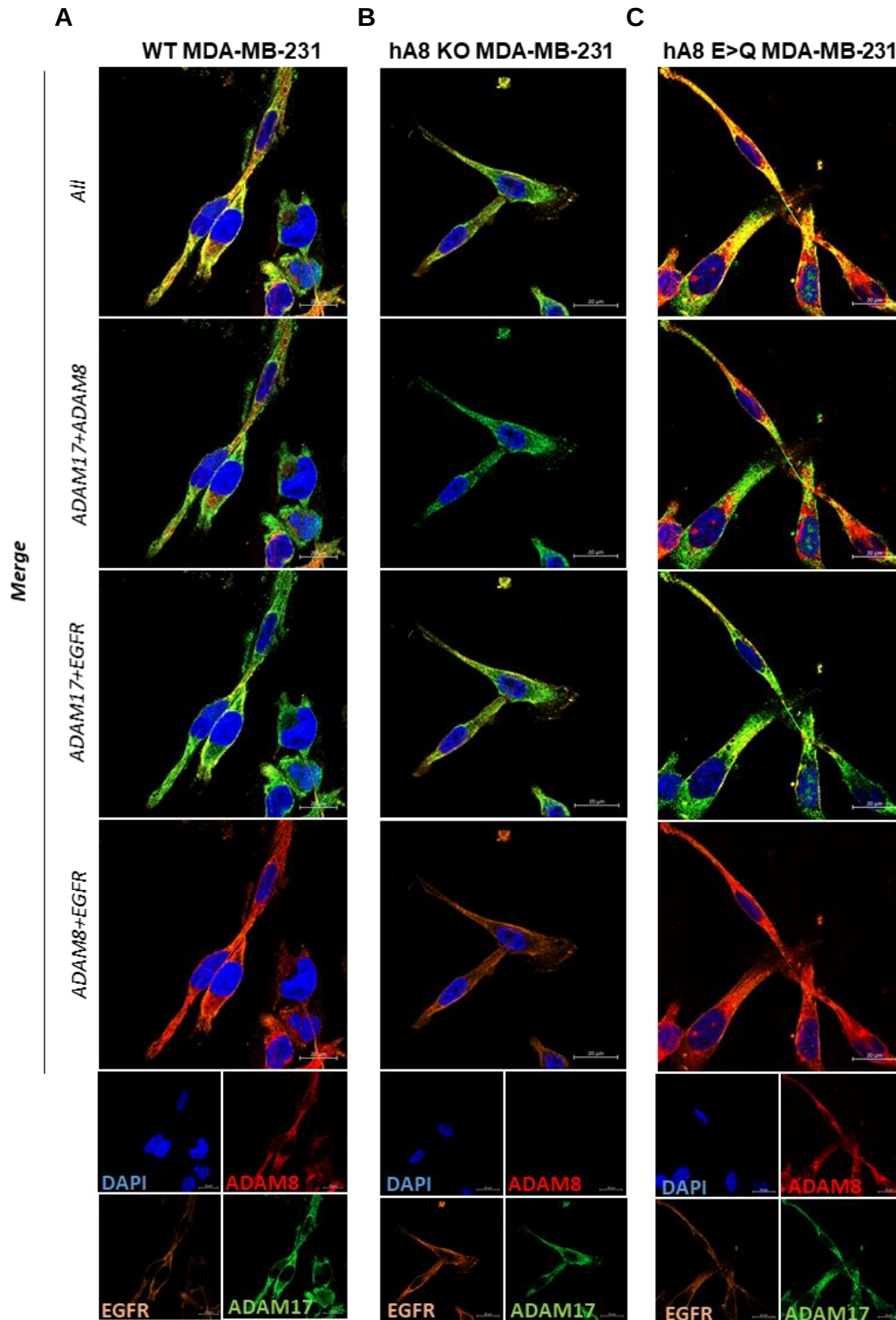


Figure 8: Cell surface colocalization of ADAM8 and ADAM17 visualized by immunofluorescence microscopy

(A, B, C) Immunofluorescence staining of endogenous ADAM17 (green) and ADAM8 (red) to determine the cell surface presence and colocalization of ADAM17 and ADAM8 in comparison to the EGFR (orange). WT, hA8 KO, and hA8 E>Q MDA-MB-231 cells were permeabilized and ADAM17 was detected using the antibody against its C-terminus (Ab19027), and ADAM8 (PA5-47047) was detected by an antibody against its ectodomain. The nucleus was stained with DAPI (scale bar, 20 μ m). (A, B, C) The localization of ADAM17 on the cell surface in WT and hA8 KO and hA8 E>Q cells was validated with confocal imaging in the presence of EGFR (orange). (C) Low detection of ADAM8 on the cell surface was observed when ADAM8 is catalytically inactive.

In parallel, to confirm the cell surface expression of ADAM17, cell surface biotinylation was performed and the cell surface proteins were detected via western blotting. Data obtained after the biotinylation revealed a similar profile for the cell surface presence of ADAM17. Mature ADAM17 was found on the cell surface in all approaches, however, no cell surface ADAM8 could be detected at the endogenous levels (Figure 9). Additionally, an overall low expression of ADAM17 and ADAM8 in hA8 E>Q cells could be seen in western blots for lysate control and consequently low mature ADAM17 on the cell surface as compared to the WT and ADAM8 KO cells. Together with the data obtained from the immunofluorescence images and the cell surface biotinylation, the inactivation or absence of ADAM8 does not affect the localization of ADAM17 on the cell surface, but with catalytically inactive ADAM8, most of ADAM8 gets retained in the secretory pathway.

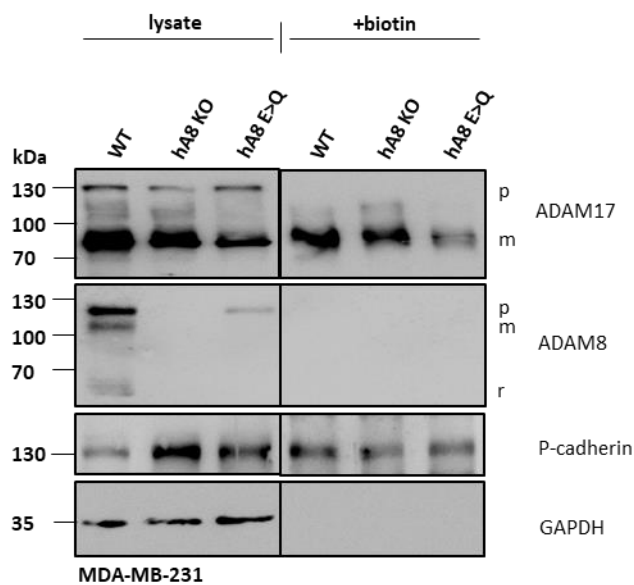


Figure 9: Cell surface protein biotinylation in MDA-MB-231 cells

Cell surface biotinylation confirming the presence of mature ADAM17 on the cell surface of WT, hA8 KO, and hA8 E>Q MDA-MB-231 cells. The endogenous cell surface proteins were biotinylated and pulled down by magnetic streptavidin beads followed by analysis via immunoblotting. ADAM17 was detected using the antibody against its disintegrin domain, in lysate controls, the pro-form of ADAM17 (p) was observed at ~130 kDa, and mature ADAM17 was detected at ~75kDa in the control and biotinylated cells. ADAM8 was only detected in the lysate control with an antibody against its ectodomain. In the untreated lysate samples the pro-form (p) of ADAM8 was detected at ~120 kDa, mature form (m) at ~100 kDa, and the remnant form (r) at ~60kDa. Pan-cadherin (p-cadherin) and GAPDH served as biotinylation and loading control, respectively.

3.1.2 Colocalization of ADAM17 and ADAM8 in the secretory pathway

Since ADAM8 is known to autocatalytically remove its pro-domain within the trans-Golgi-network (Schlomann et al., 2002), a possibility of shedding its substrates already in the secretory pathway exists. A study was conducted to observe the shedding of ADAM17 in ADAM10/17^{-/-} HEK293T cells overexpressing mADAM8 and mADAM17, with a membrane non-permeable ADAM8-specific cyclic peptide inhibitor (BK1361) and membrane permeable batimastat (BB94) (Scharfenberg et al., 2020). A decreased shedding of ADAM17 was observed only in the presence of BB-94 implying the interaction of ADAM8 and ADAM17 in the secretory pathway (Scharfenberg et al., 2020). The colocalization and hence the interaction of ADAM17 and ADAM8 on the cell surface is evident from immunofluorescence images. However, the intracellular staining of ADAM8 and ADAM17 was also observed. Therefore, to validate the early intracellular colocalization and hence the interaction of ADAM17 and ADAM8, all variants of MDA-MB-231 (WT, hA8 KO, and hA8 E>Q) cells were also analyzed via immunofluorescence microscopy in the presence of an ER marker, ERp5. After reaching the desired confluency of 80%, the cells were permeabilized with 0.2% saponin and were immuno-stained with an antibody against ERp5 (orange), an antibody against the C-terminus of ADAM17 (Ab19027, 1:500 in 10% FCS in PBS), and antibody raised against the ectodomain of ADAM8 (PA5-47047, 5 µg/mL in 10% FCS in PBS). For the WT cells, most of ADAM8 (red) seemed to be equally distributed in the ER and the later secretory pathway. ADAM17 (green) was moderately co-localized with ADAM8 within the ER and in the later secretory pathway (Figure 10A). In the absence of ADAM8 low amounts of ADAM17 were detected within the ER as compared to the other cells and most of the signal was observed in the later secretory pathway or at the plasma membrane (Figure 10B). In the cells with inactive hADAM8 (hA8 E>Q), most of the ADAM8 seems to be retained within the secretory pathway and ADAM17 was again moderately colocalized with ADAM8 within the ER but also observed outside the ER like in WT cells (Figure 10C). This suggested that the inactivation of ADAM8 does not influence the interaction of the two proteins within the secretory pathway. The immunofluorescence images confirmed the commencement of protein-protein interaction between ADAM8 and ADAM17 within the early secretory pathway.

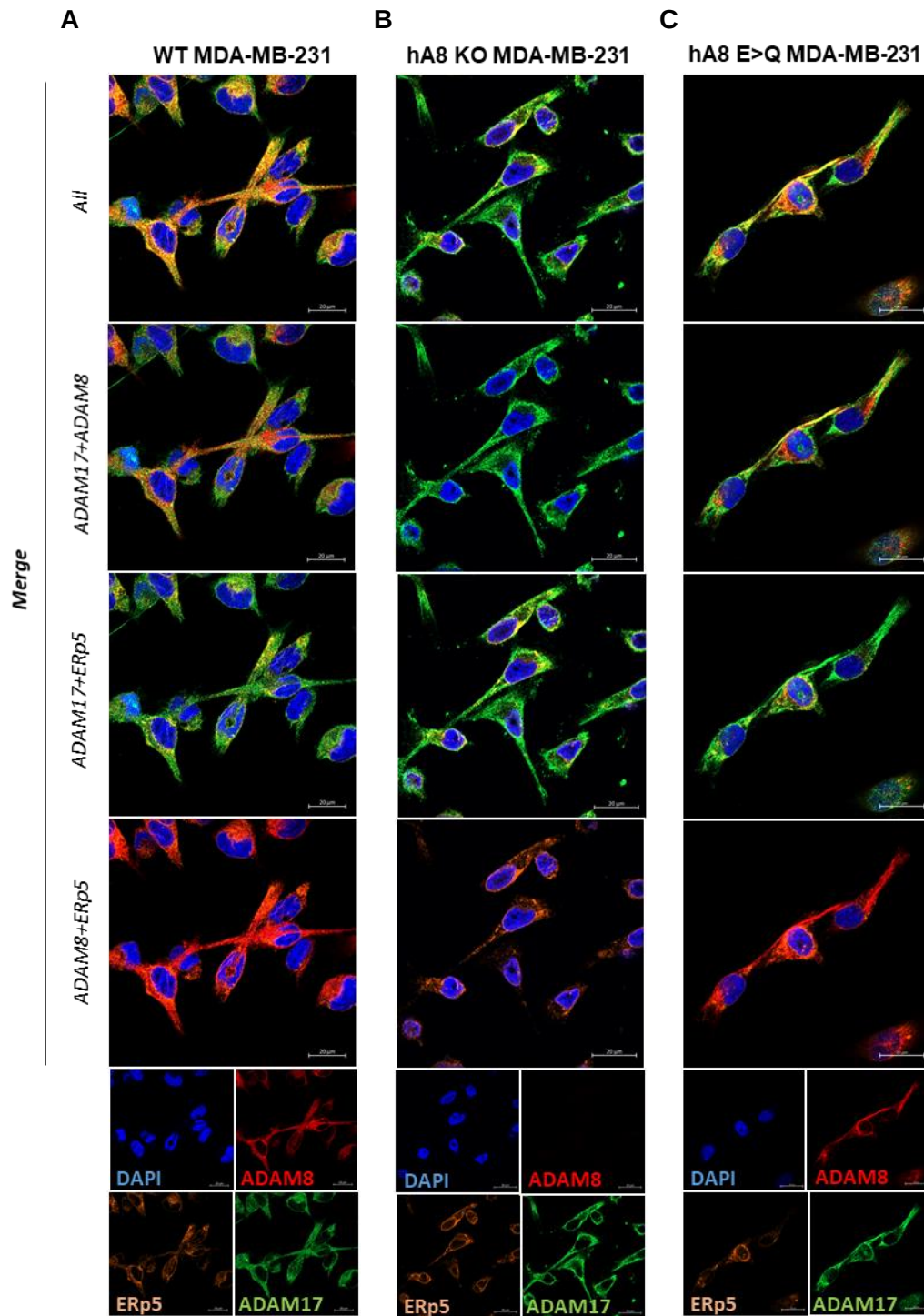


Figure 10: Colocalization of ADAM8 and ADAM17 in the secretory pathway

(A, B, C) Immunofluorescence staining of endogenous ADAM17 (green), ADAM8 (red), and ER marker ERp5 (orange). WT, hA8 KO, and hA8 E>Q MDA-MB-231 cells were permeabilized and ADAM17 was detected using the antibody against its C-terminus (Ab19027), and ADAM8 was detected by an antibody against its ectodomain (PA5-47047). The nucleus was stained with DAPI (scale bar, 20 μm). (A, C) Merge image (second from top) for green and red channels showing colocalization of ADAM17 and ADAM8 within the ER and later secretory pathway.

3.1.3 Shedding of ADAM17 in the presence of protease or trafficking inhibitors

According to immunofluorescence images, ADAM8 and ADAM17 are already observed to be colocalized in the secretory pathway apart from the cell surface. As the interaction of ADAM8 and ADAM17 occurs throughout the secretory pathway the proteolytic release of soluble ADAM17 by ADAM8 might also occur intracellularly. To further figure out where the shedding of ADAM17 commences, the MDA-MB-231 cells were analyzed for the proteolytic release of the ectodomain of ADAM17 via western blotting in the absence or presence of protease or protein-trafficking inhibitors.

By this approach, the shedding can be blocked at different locations. To inhibit the shedding at the cell surface, cells were supplied with EDTA (a metal chelator and a non-permeable metalloprotease inhibitor). Within the secretory pathway, the inhibition was done by applying 1,10-phenanthroline (1,10 phe.) which is a membrane permeable metal chelator. Finally, to disrupt the ER to Golgi trafficking of the proteins, the cells were treated with Brefeldin A (BFA) (Klausner, March 1992).

Since the western blot detection of endogenous shed proteins in the supernatant is challenging, in a parallel setup, cells transiently transfected with human ADAM17 were also analyzed for the shedding. The cells were starved for 12-24 h in serum-free media with or without the inhibitors. Figure 11 depicts the predicted outcome for the shedding influenced by inhibitors. In the case of EDTA and 1,10-phenanthroline, the cells were treated for 24 h with the inhibitors in the serum-free medium. For BFA, the cells were incubated for 12 h from starvation.

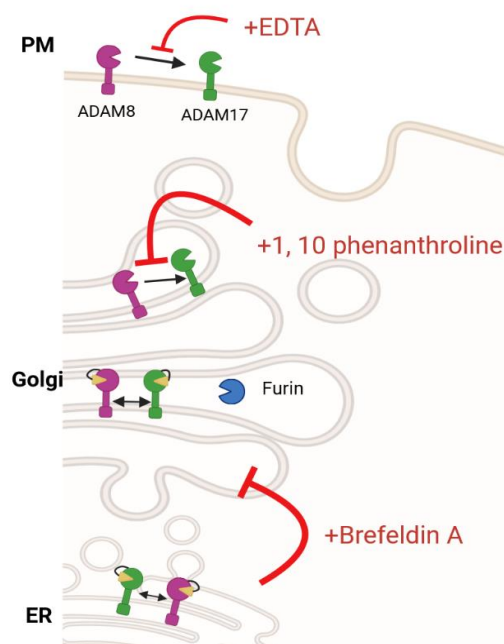


Figure 11: Predicted outcome for the shedding of ADAM17 in the presence of inhibitors

Cartoon for inhibition of ADAM8 dependent shedding in the presence of inhibitors in WT MDA-MB-231 cells. From bottom to top: Brefeldin A disrupts the ER to Golgi trafficking; 1,10-phenanthroline is cell permeable metalloprotease inhibitor; EDTA is a non-permeable, general matrix metalloprotease inhibitor.

According to the immunoblots, in the case of endogenous and overexpressed ADAM17, the levels of sADAM17 in the cell supernatant were unaffected in the presence of EDTA, in contrast, the levels only decreased in the presence of 1,10-phenanthroline. In the case of BFA, the effect was not very clear for the endogenous ADAM17 (Figure 12A). However, in cells with overexpressed ADAM17, the shedding was depleted by 1,10-phenanthroline and BFA, suggesting that the shedding of ADAM17 mainly takes place in the secretory pathway (Figure 12A). The corresponding lysate blots for ADAM17 also show an overall reduction in the expression of pro (~130 kDa) and mature ADAM17 (~80 kDa) in the presence of 1,10-phenanthroline and BFA (Figure 12A). Since ADAM8 is identified as one of the sheddases of ADAM17, sADAM17 may also be generated by other proteases and autoproteolytically. In that line, the shedding of ADAM17 in the absence of ADAM8 was also analyzed. In hA8 KO cells, little to no endogenous sADAM17 was detected. However, upon overexpression of ADAM17, sADAM17 was detected in the cell supernatant and ADAM8 independent shedding appeared to decrease only in the presence of 1,10-phenanthroline and slightly in the presence of EDTA. DMSO and BFA treatment did not differ in terms of secretion of shed ADAM17 with overexpressing hADAM17. In the lysate blots for ADAM17, only the expression of pro (~130 kDa) ADAM17 was reduced in the presence of BFA (Figure 12B). Although the shedding was also observed in the absence of ADAM8, as compared to the WT cells, the shedding in hA8 KO cells was less prominent. However, there was considerable shedding when hADAM17 was overexpressed and inhibited by 1,10-phenanthroline and with BFA (Figure 12C).

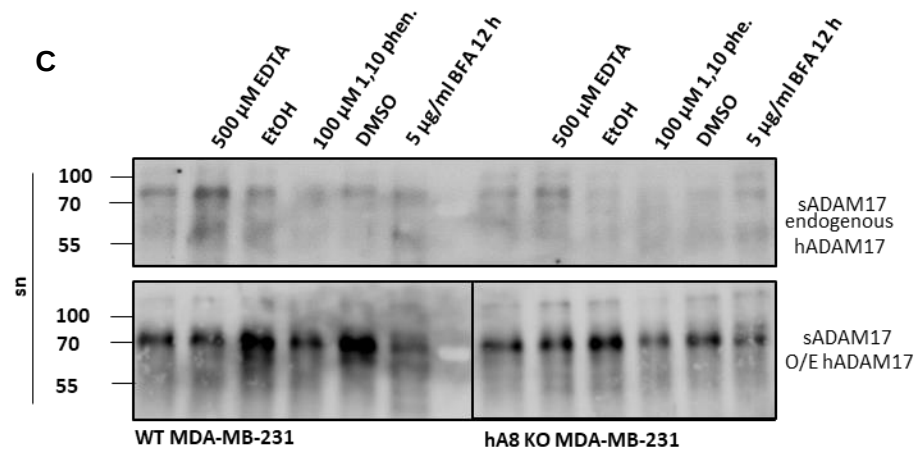
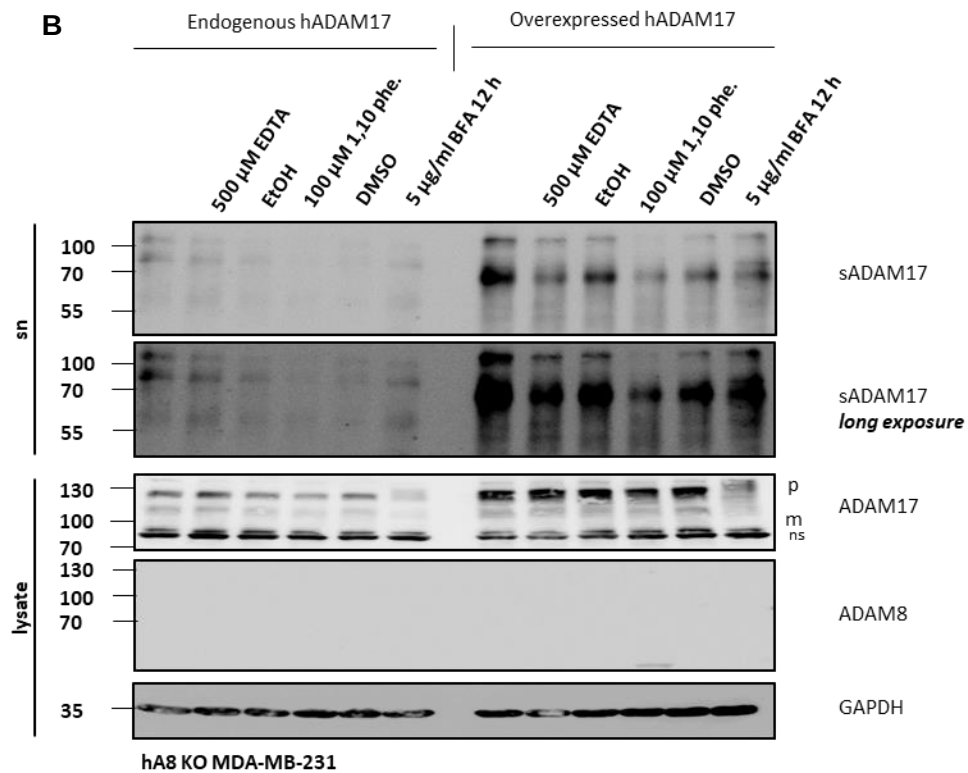
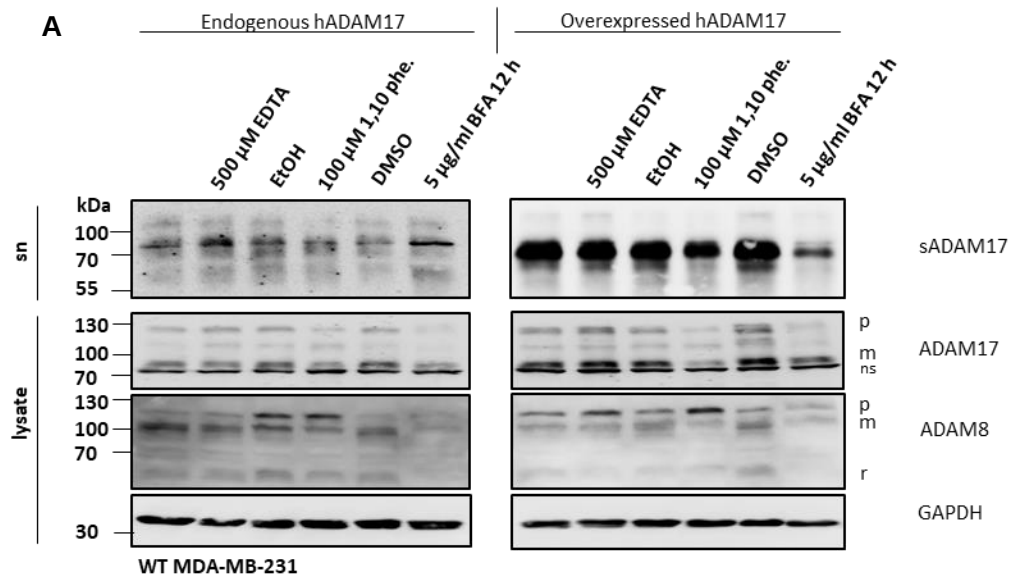


Figure 12: Shedding of endogenous and overexpressed human ADAM17 in the presence of inhibitors

WT and hA8 KO MDA-MB-231 cells were transfected with pcDNA3.1 or hADAM17 and cells were starved in serum-free media with or without inhibitors. The shedding of ADAM17 was detected via western blots in the cell supernatant. The cells were incubated for 24 h with EDTA or 1,10-phe. and for 12 h with BFA from the time of starvation. In the lysates, pro and mature forms of ADAM17 and ADAM8 are marked as 'p' and 'm': and the remnant form of ADAM8 is marked with 'r'. (A) Western-blot analysis for the shedding of endogenous and overexpressed hADAM17 in WT cells with inhibitors and their respective controls: EDTA (500 μ M), 1,10-phe. (100 μ M), and BFA (5 μ g/ml). (B) Western-blot analysis for the shedding of endogenous and overexpressed hADAM17 in hA8 KO cells with inhibitors and their respective controls: EDTA (500 μ M), 1,10-phe. (100 μ M), and BFA (5 μ g/ml). (C) Comparative western-blots of the supernatants from WT and hA8 KO cells for the shedding of endogenous and overexpressed hADAM17. The compared supernatant samples belonged to the same experiments as A and B for WT and hA8KO cells respectively.

Controls: for EDTA- milli-Q, for 1,10-phenanthroline- EtOH, for BFA- DMSO

Non-specific bands in lysates are denoted with 'ns'.

3.1.4 Proteolytically released soluble ADAM17-mediated peptide processing

As observed from the western blots, the shedding of endogenous and overexpressed ADAM17 was not affected in the presence of EDTA. However, particularly in the presence of 1,10-phenanthroline and BFA, the shedding was affected. Additionally, an ADAM8 independent shedding of ADAM17 was observed in hA8 KO cells, therefore, the basal sADAM17 activity in the supernatants of WT and hA8 KO cells was determined (Figure 13A). As observed earlier, in the absence of ADAM8, the release of sADAM17 is noticeably low, and the activity assay aligns with the signal on the blots (Figure 12C, 13A). Next, the supernatants obtained from the shedding inhibition study were further assessed for the proteolytic activity of the released soluble ADAM17 against an ADAM17-specific fluorogenic peptide (PEPDAB64). Each sample was analyzed for ADAM17-specific activity with or without competitive hydroxamate inhibitor, GW to block peptide cleavage and autocatalytic processing of ADAM17. Even though the activity profile for endogenous sADAM17 lacks statistical significance, a trend can be observed where in the WT cells, the activity is low due to less sADAM17 secreted by cells treated with 1,10 phenanthroline or BFA (Figure 13B). As observed from the blots, ADAM8 independent shedding decreased in the presence of 1,10-phenanthroline. In the same line, low activity due to less sADAM17 was noted (Figure 13C). Similarly, in the case of soluble ADAM17 obtained from shedding of overexpressed hADAM17, the proteolytic activity was barely affected in the supernatant of cells treated with EDTA but decreased significantly in the presence of membrane permeable inhibitors 1,10 phenanthroline and BFA (Figure 13D) which is in line with the immunoblots for the shed and overexpressed ADAM17 (Figure 12A). Notably, in comparison to the WT cells, sADAM17 generated in the hA8 KO cells showed a nearly similar trend in proteolytic activity (Figure 13D, E). However, BFA did not influence ADAM8 independent shedding of ADAM17. Taken together, the generation of proteolytically active

soluble ADAM17 in the presence or absence of ADAM8 suggests that ADAM8 is not the only sheddase of ADAM17, however, it is one of the sheddases of ADAM17, active in the secretory pathway and on the cell surface.

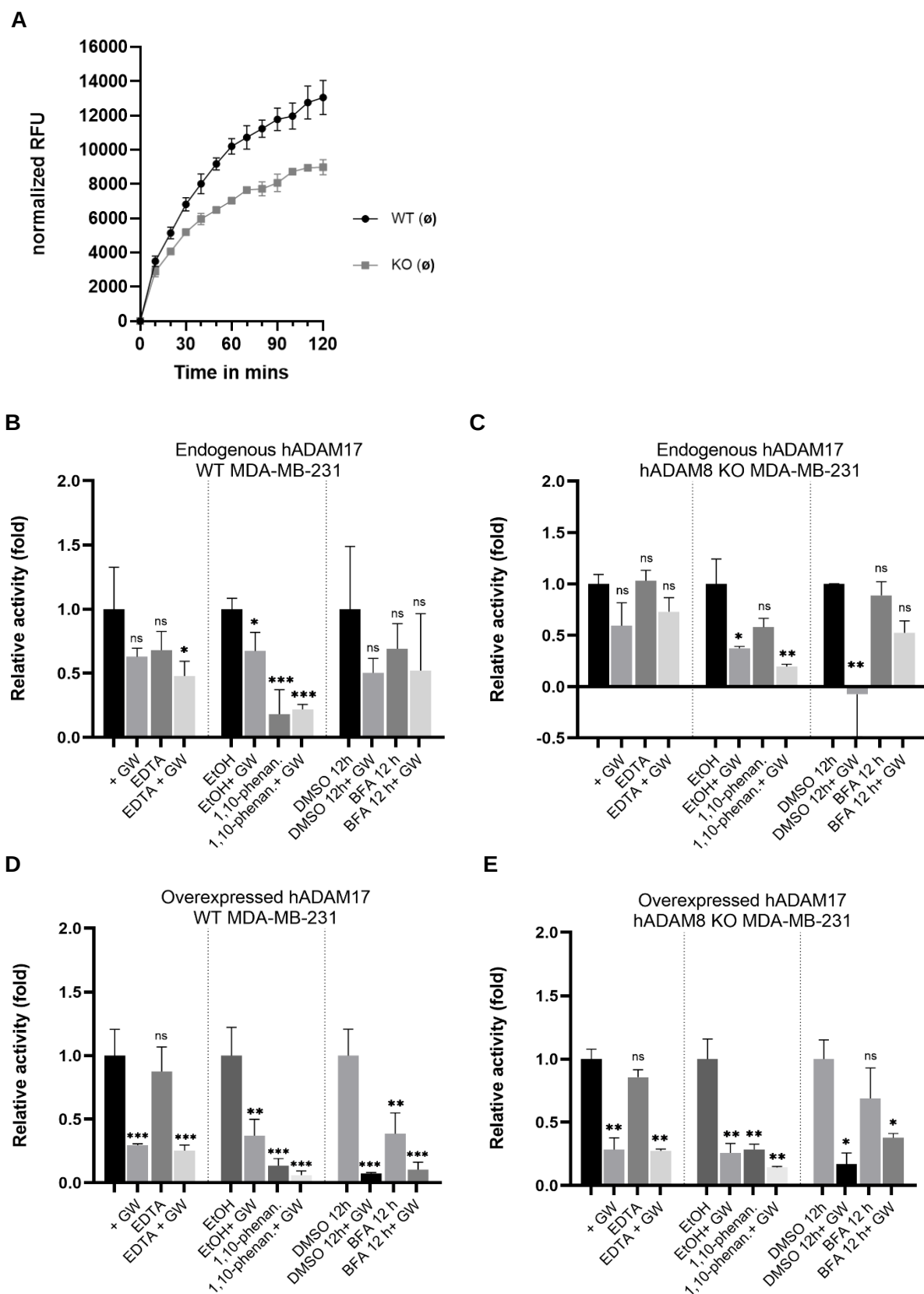


Figure 13: Proteolytic activity of shed hADAM17

WT and hA8 KO MDA-MB-231 cells were transfected with empty vector or hADAM17 and cells were starved in serum-free media with or without inhibitors. The cell supernatants were collected after 24 hours in the case of EDTA and 1,10-phenanthroline and after 12 hours in the case of BFA from the time of starvation and inhibitor addition. The activity was analyzed in freshly harvested and cleared cell supernatants. (A) Activity analysis of endogenous sADAM17 in the supernatant from WT and hA8KO MDA-MB-231 cells against fluorogenic peptide PEPDAB64 (an ADAM17 specific peptide); n=2 technical replicates (B, C) Activity analysis for endogenous sADAM17 obtained from inhibitor-treated cells. WT and hA8 KO MDA-MB-231 cell supernatants were analyzed against the fluorogenic peptide (PEPDAB64) in the presence or absence of GW (3 μ M) at 60 minutes. (D, E) Activity analysis for sADAM17 released from inhibitor-treated WT and hA8 KO cells overexpressing hADAM17. The supernatants were analyzed against the fluorogenic peptide (PEPDAB64) in the presence or absence of GW (3 μ M) at 60 minutes.

The activity of each control was set to 1 and relative proteolytic activity in folds was analyzed via graph pad prism (n=3 biological replicates for WT and n=2 technical replicates for ADAM8 KO cells), data represented as mean $\pm$ SD, and statistical analysis was performed by one-way ANOVA followed by Dunnett's post hoc test (*: p<0.05; **: p<0.01; ***: p<0.001).

3.2 Shedding of ADAM17 in the presence or absence of iRhom1/2

iRhom1 and iRhom2 are crucial for the trafficking and regulation of the membrane-bound ADAM17 (Christova et al., 2013; Dulloo et al., 2019), therefore it was interesting to understand whether iRhoms play any role in terms of regulation of the shedding of ADAM17. Since in the majority of breast cancer cells including MDA-MB-231 cells, the expression of particularly iRhom1 is upregulated (Canzoneri et al., 2014; Yan et al., 2008; Zhou et al., 2014), there could be a possible involvement of iRhom1 in the observed shedding of ADAM17. However, the involvement of either of the iRhoms is yet to be investigated. Hence, to analyze the shedding of mADAM17 available WT mEF cells, known to endogenously co-express iRhom1 and 2 were initially compared to iRhom1/2 knock-out (iRhom1/2^{-/-}) and iRhom2 stably transfected mEF cells. The analysis for the iRhom-dependent or independent shedding of mADAM17 was performed via immunoblotting.

3.2.1 Generation of stably transfected mEF cells

Since iRhom2 is prevalently involved in the regulation of ADAM17 and its activity, the role of iRhom2 and its phosphorylation was also investigated regarding the shedding of ADAM17. The phosphorylation of the N-terminal tail of iRhom2 plays a major role in the regulation of ADAM17's shedding activity, however, this mechanism does not play any role in the trafficking and maturation of ADAM17 (Cavadas et al., 2017; Grieve et al., 2017). The question was whether the phosphorylation-induced separation of ADAM17 from iRhom2 makes ADAM17 more susceptible to shedding. Therefore, to investigate the phosphorylation aspect, stably transfected mEF cells with iRhom2 (iR2_HA), iRhom2 dead (iR2_dead_HA; mutant with no

phosphorylation ability), and iRhom2 dead R18 (iR2_dead_R18_HA; mutant with rescued phosphorylation at N-terminal tail) were generated in iRhom1/2^{-/-} mEFs (Figure 14A). The expression analysis was then confirmed via western blots (Figure 14B).

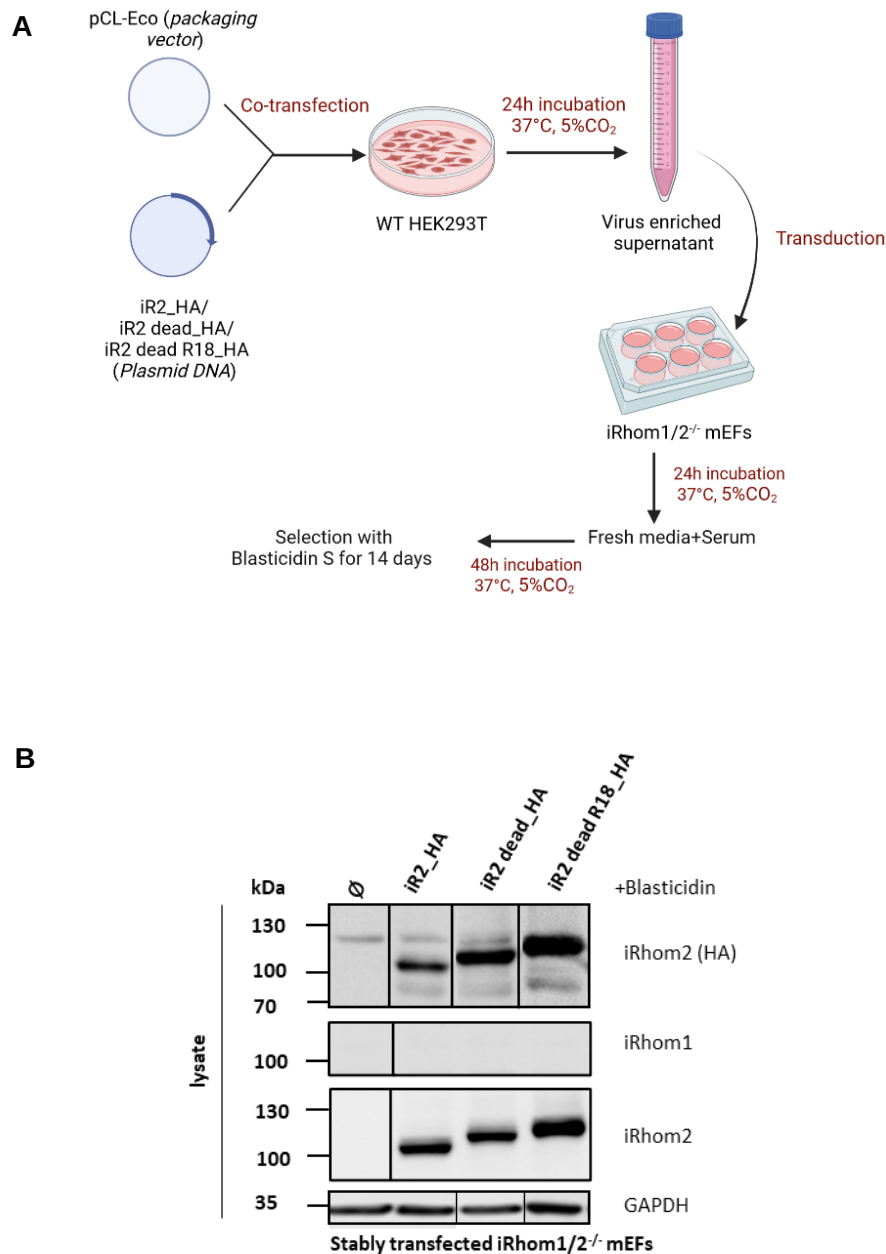


Figure 14: Stable transfection of iRhom1/2^{-/-} mEF cells

To assess the influence of iRhom2 phosphorylation, iRhom1/2^{-/-} mEF cells were stably transfected. (A) A scheme for stable transfection is shown (B) Expression analysis for iRhom2 variants in stably transfected mEF cells via western blot. iRhom2 was detected via HA-tag antibody (C29F4) and iRhom2 specific antibody in stably transfected mEF cells with iRhom2 (iR2_HA), iRhom2 dead (iR2_dead_HA; mutant with no phosphorylation ability), and iRhom2 dead R18 (iR2_dead_R18_HA; mutant with rescued phosphorylation N-terminal tail). iRhom1 served as loading control and GAPDH served as positive control.

3.2.2 Generation and characterization of murine ADAM17 with HA-tag after pro-domain

To investigate the shedding of ADAM17, an overexpression plasmid for membrane-bound murine ADAM17 was constructed with an HA-tag after the pro-domain and a flag tag at the cytoplasmic C-terminal. The HA-tag was introduced C-terminal to the furin cleavage side (RVKR), to verify the removal of the pro-domain of released ectodomain in the absence or the presence of iRhoms. To characterize the construct, ADAM10/17^{-/-} HEK293T cells were transiently transfected with wild-type murine ADAM17 (mADAM17 WT) and mADAM17 PD_HA plasmid and the expression was analyzed via western blots (Figure 15A). ADAM17 in the lysates and supernatant was detected by applying an antibody raised against ADAM17 ectodomain (10.3), HA-tag (C29F4), and flag-tag (M2). In the lysate blots, for mADAM17 PD_HA, the pro-form (p) of ADAM17 was detected by anti-ADAM17, anti-HA-tag and anti-flag antibodies. For mADAM17 WT, the pro-form was detected via an anti-ADAM17 antibody (Figure 15A). In the ultracentrifuged and TCA precipitated supernatant (*sn), the released ectodomain was successfully identified with anti-ADAM17 and anti-HA-tag, confirming the release of the mature ectodomain without the pro-domain. As expected, no ADAM17 was detected in the supernatant with the anti-flag-tag antibody suggesting complete removal of the C-terminal domain. Furthermore, to ensure the presence of mature ADAM17 on the cell surface, biotinylation of the cell surface proteins was carried out in ADAM10/17^{-/-} HEK293T cells transfected with mADAM17 WT and mADAM17 PD_HA. The pro-form (p) and the mature form (m) of ADAM17 were detected for both the constructs upon biotinylation confirming the presence of mature ADAM17 on the cell surface. (Figure 15B). Next, the proteolytic activity of the shed ADAM17 was determined in the cell supernatant, against the fluorogenic TNF- α based peptide. The sADAM17 in the ultracentrifuged supernatants displayed proteolytic activity which was inhibited significantly in the presence of the competitive hydroxamate inhibitor GW, (Figure 15C).

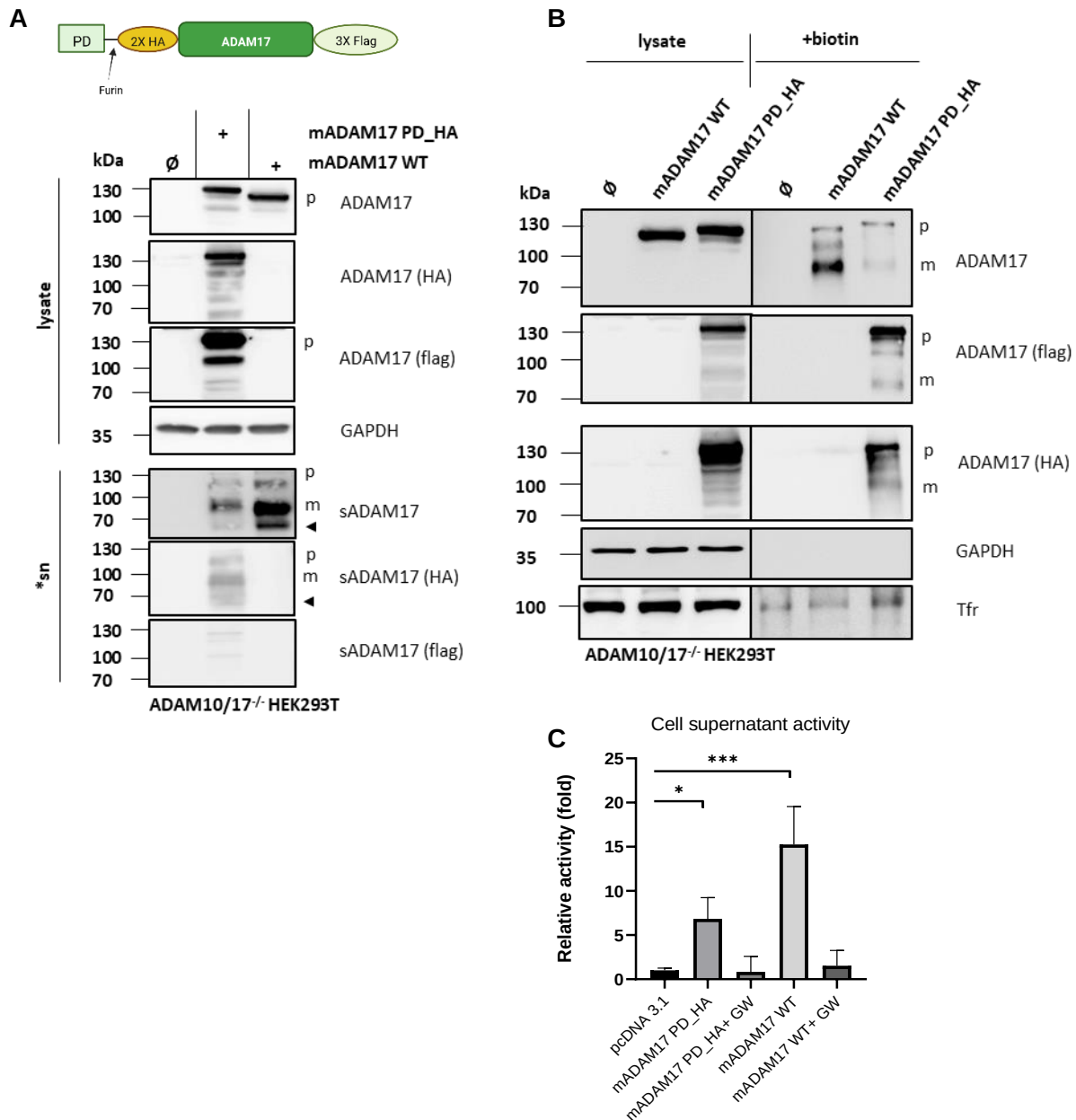


Figure 15: Biochemical properties of murine ADAM17 with HA-tag C-terminal of the pro-domain

(A) Cartoon representing the membrane-bound murine ADAM17 with N-terminal HA-tag after the pro-domain (mADAM17 PD_HA) and its transfection in ADAM10/17^{-/-} HEK 293T cells followed by western blot analysis to assess its expression and shedding of ADAM17 in the ultra-centrifuged cell supernatant (*sn) in comparison with wild-type murine ADAM17 (mADAM17 WT). ADAM17 in lysates and supernatants was detected via anti-ADAM17 (10.3), anti-HA-tag (C29F4), and anti-flag (M2) antibodies. GAPDH served as lysate positive control. The pro and mature forms of ADAM17 are marked with (p) and (m) respectively. A mature, further processed sADAM17 is marked with a black arrowhead. (B) Cell surface biotinylation in ADAM10/17^{-/-} HEK 293T cells transfected with mADAM17 WT and mADAM17 PD_HA displaying pro (p) and mature (m) form in the cell-lysate control and biotinylated cell surface ADAM17. Transferrin (Tfr) and GAPDH served as biotinylation and loading control, respectively. (C) The activity of proteolytically released soluble ADAM17 in ultracentrifuged cell supernatant in the presence or absence of GW (3 μ M) against TNF- α based fluorogenic peptide in relative fluorescence unit (RFU), n=3 technical replicates. The data is represented as mean $\pm$ SD and statistical analysis was performed by one-way ANOVA followed by Dunnett's post hoc test (*: p<0.05, **: p<0.01; ***: p<0.001).

In addition to the characterization of mADAM17 PD_HA in ADAM10/17^{-/-} HEK 293T cells, the cell surface expression was also examined, in iRhom1/2^{-/-} mEF cells. The detection of ADAM17 in the lysate and the biotinylated sample was done by applying ADAM17 specific antibody (10.3), an antibody against HA-tag, and an antibody against flag-tag. As expected for mADAM17 WT transfected cells, no mature ADAM17 was detected. On the other hand, in the case of biotinylated mADAM17 PD_HA, ADAM17 specific antibody detected the mature ADAM17 at ~90 kDa apart from the pro-form (~130 kDa). However, using an antibody against HA and flag-tag, only the pro-form of ADAM17 was detected (Figure 16).

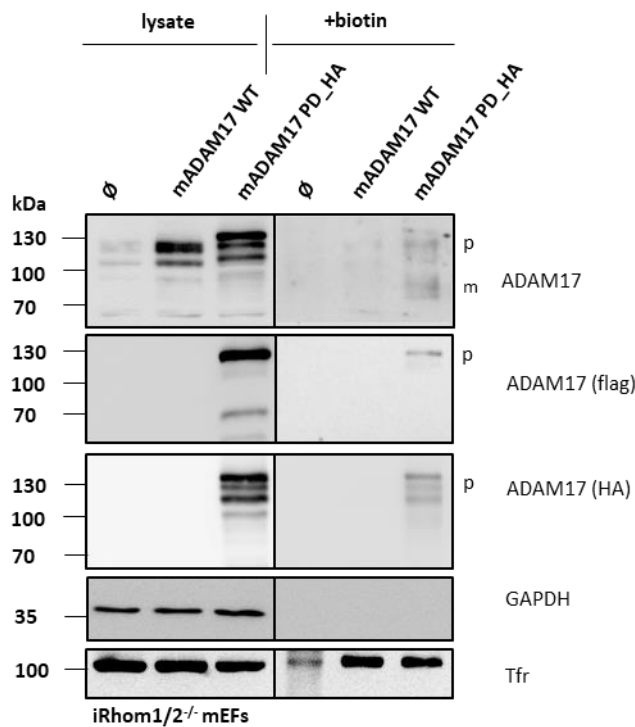


Figure 16: Biotinylation of cell surface ADAM17 in iRhom1/2^{-/-} mEFs

Cell surface biotinylation in iRhom1/2^{-/-} mEFs transfected with mADAM17 WT and mADAM17 FL PD_HA. ADAM17 in lysate control and biotinylated sample was analyzed by anti-ADAM17, anti-HA-tag and anti-flag-tag antibodies. The signal for pro-form (~130 kDa) and mature form (~85 kDa) are marked as (p) and (m) respectively. Transferrin (Tfr) and GAPDH served as biotinylation and loading control, respectively.

3.2.3 Impact of iRhom2 and its phosphorylation on the shedding of ADAM17

Here, the WT, iRhom1/2^{-/-} and iRhom2 stable transfected mEFs were transiently transfected with pcDNA3.1, mADAM17 WT, and mADAM17 PD_HA constructs and analyzed for the shedding of ADAM17. Post transfection, the cells were starved in serum-free media for 24 h. The cells were harvested, and the supernatant was collected to analyze the sADAM17 in the cell supernatant (Figure 17A). The endogenous sADAM17 released by mock-transfected cells showed no difference in shedding intensities between WT and iRhom2 stable transfected (iR2_HA) mEFs but a slightly low shedding in iRhom1/2 double knockout cells was observed. On the other hand, the shedding of overexpressed variants of membrane-bound ADAM17 was slightly higher in the absence of iRhom1/2 but did not show any variance between WT and iR2_HA mEFs (Figure 17A). The overexpression of iRhom2 did not make an impact on the shedding of ADAM17. In the lysates, the pro and mature forms of ADAM17 were only detected in WT and iR2_HA mEFs applying anti-ADAM17 antibody. Anti-HA-tag antibody detected the pro-form of ADAM17. The signal for the HA-tag in the iR2_HA mEFs at ~100 kDa appeared due to the HA-tag of iRhom2 (Figure 17A). Upon testing the shedding of endogenous ADAM17 and overexpressed mADAM17 PD_HA in phosphorylation mutants of iRhom2, again high shedding (supernatant blots for HA-tag) in the absence of iRhom1/2 was observed in comparison to other cases (Figure 17B). Notably, in the lysate blot, the overall expression of ADAM17 in the WT cells appeared to be lower than in the other cells. No mature form of ADAM17 was observed in the lysates in the absence of iRhom1/2. Taken together, iRhoms do not play a significant role in the shedding of ADAM17, however, it is possible that in the absence of iRhom1/2, ADAM17 is more accessible to its sheddases.

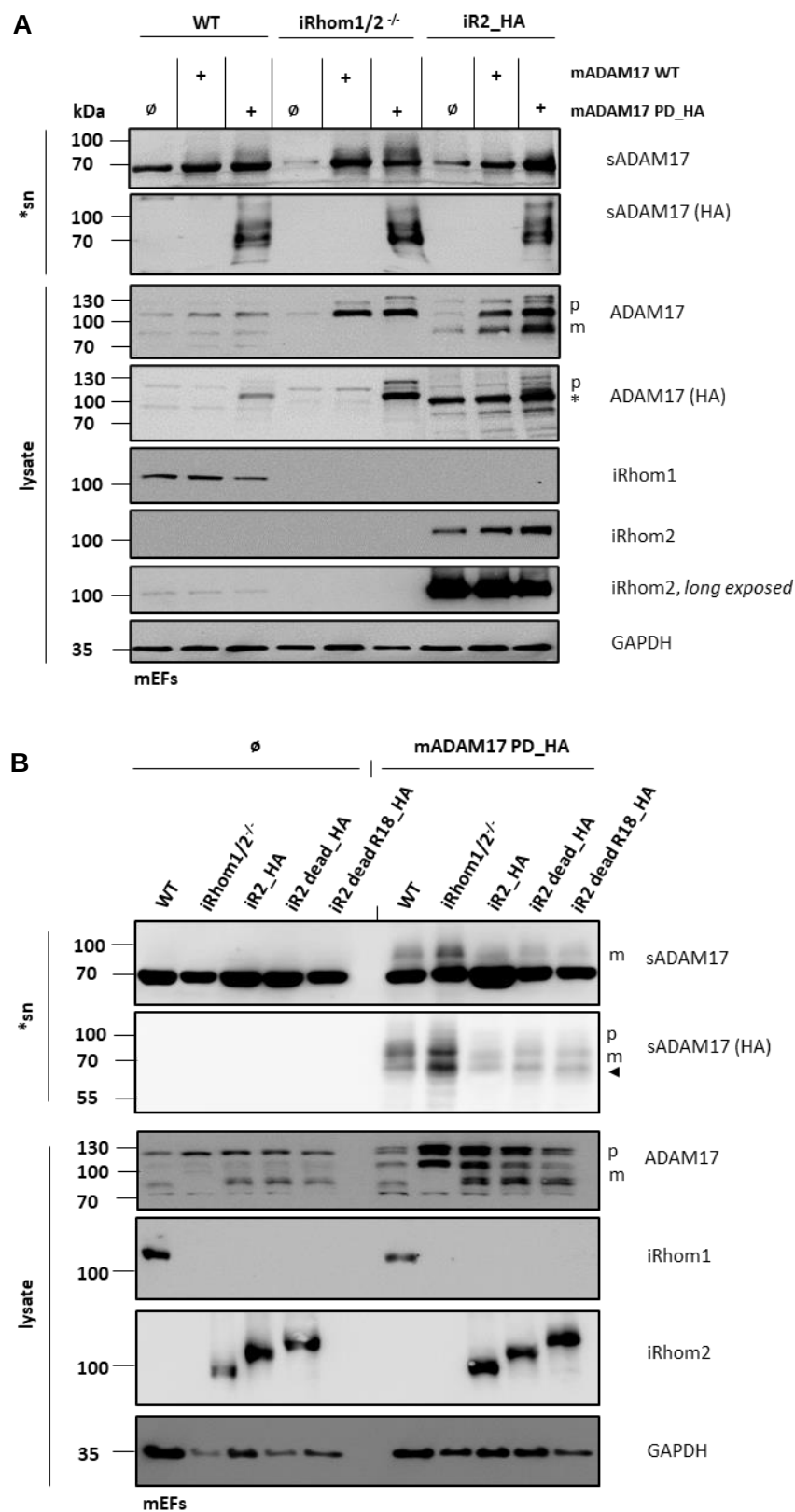


Figure 17: iRhom2 and its phosphorylation independent shedding of ADAM17

The influence of iRhom2 phosphorylation was examined in murine embryogenic fibroblasts (mEFs). The cells were transfected with pcDNA3.1, mADAM17 WT, and mADAM17PD_HA, and thereafter the cell supernatant and lysates were analyzed after 24h from starvation in serum-free media. ADAM17 was detected by anti-ADAM17 (10.3) and anti-HA-tag (C29F4) antibodies; iRhom1 and iRhom2 were detected by specific anti-iRhom1 and anti-iRhom2

antibodies and GAPDH served as loading control. (A) Western blot analysis for the shedding of mADAM17 in WT and iRhom1/2^{-/-} mEFs and mEFs stably expressing iRhom2 (iR2_HA), transfected with empty vector, mADAM17 WT, and mADAM17 PD_HA constructs. (B) Western blot analysis for the shedding of mADAM17 in WT mEF and iRhom1/2^{-/-} mEF and mEF cells stably expressing iR2_HA, iR2 dead_HA, iR2 dead R18_HA, transfected with empty vector and mADAM17 PD_HA constructs.

Soluble ADAM17 (~70 kDa) was analyzed in cleared, ultra-centrifuged cell supernatant by employing the murine ADAM17 specific antibody (10.3) and anti-HA antibody. The pro (p) (~130 kDa) and mature (m) (~85 kDa) ADAM17 was only detected in the lysates of WT mEFs cells and mEFs stably transfected with iRhom2 and pro (p) form was detected in iRhom1/2^{-/-} mEFs. (*) signifies the 100 kDa signals for iRhom2_HA in stably transfected mEFs.

(*sn): supernatants were ultracentrifuged and TCA-precipitated.

3.3 Shedding functions of soluble ADAM17

The *in vitro* studies performed employing rec. sADAM17 provided insights into the potential role of sADAM17 within the protease web via processing various soluble substrates like cystatin-C or PCPE1 (Scharfenberg et al., 2020). In the same study, a known membrane-bound ADAM17 substrate APP695 (Buxbaum et al., 1998) was noted to be shed by the recombinant sADAM17 (Scharfenberg et al., 2020). However, further detailed analysis was yet to be done. Therefore, it was interesting to analyze the shedding properties of sADAM17 against the known substrates of membrane-bound ADAM17. To identify whether sADAM17 is also a sheddase, an overexpression construct was generated where the ectodomain of mADAM17 is N-terminally HA-tagged and C-terminally flag tagged (hereon, called HA_sADAM17_flag).

3.3.1 Characterization of soluble ADAM17

Before analyzing the shedding capacity of HA_sADAM17_flag, this construct was characterized in easy-to-manipulate ADAM10/17^{-/-} HEK293T cells. The biochemical characterization was done in terms of expression, maturation, processing, glycosylation, and trafficking of HA_sADAM17_flag. ADAM10/17^{-/-} HEK293T cells were transiently transfected with the overexpression sADAM17 plasmid and starved in serum-free media for 24 h for the release of the ectodomain into the cell supernatant. The protein expression and secretion were analyzed in cell lysates and ultracentrifuged, TCA-precipitated supernatants via western blotting. In the lysate blots, the ~110 kDa band corresponding to the pro-form (p) of sADAM17 was observed in the blots against the HA-tag, flag-tag, and the ectodomain of ADAM17. In the supernatant, the ~70 kDa band corresponds to the sADAM17 in the blots against the flag tag and ectodomain of ADAM17. For the blots against the HA-tag, the ~100 kDa band corresponds to the pro-form of ADAM17 (~100 kDa). Additionally, ~35 kDa band (anti-HA-tag blot)

corresponding to the pro-domain (PD) indicated the removal of the pro-domain and release of the mature ectodomain of ADAM17 (Figure 18A).

Thereafter, the proteolytic activity of the overexpressed sADAM17 (HA_sADAM17_flag) and the proteolytically released sADAM17 was tested. Therefore, ADAM10/17^{-/-} HEK293T cells were transfected with sADAM17 and mADAM17 WT, and the cells and supernatants were collected after 24 h from transfection. The release of sADAM17 was tested in TCA precipitated total supernatant and ultracentrifuged supernatant. To check whether the soluble ADAM17 is escaping through vesicles, the microvesicles (MV) obtained after ultracentrifuge were resuspended and TCA precipitated. The sADAM17 in the supernatant samples was analyzed via western blots (Figure 18B, C). released soluble ADAM17 was analyzed in all supernatant samples. The activity assay was performed by examining the cleavage of ADAM17 specific TNF- α based fluorogenic-peptide in the presence or absence of GW (3 μ M) and the readout was measured by spectrophotometer. The overexpressed sADAM17 and proteolytically released sADAM17 displayed comparable proteolytic activity which was efficiently inhibited in the presence of GW (18D, E).

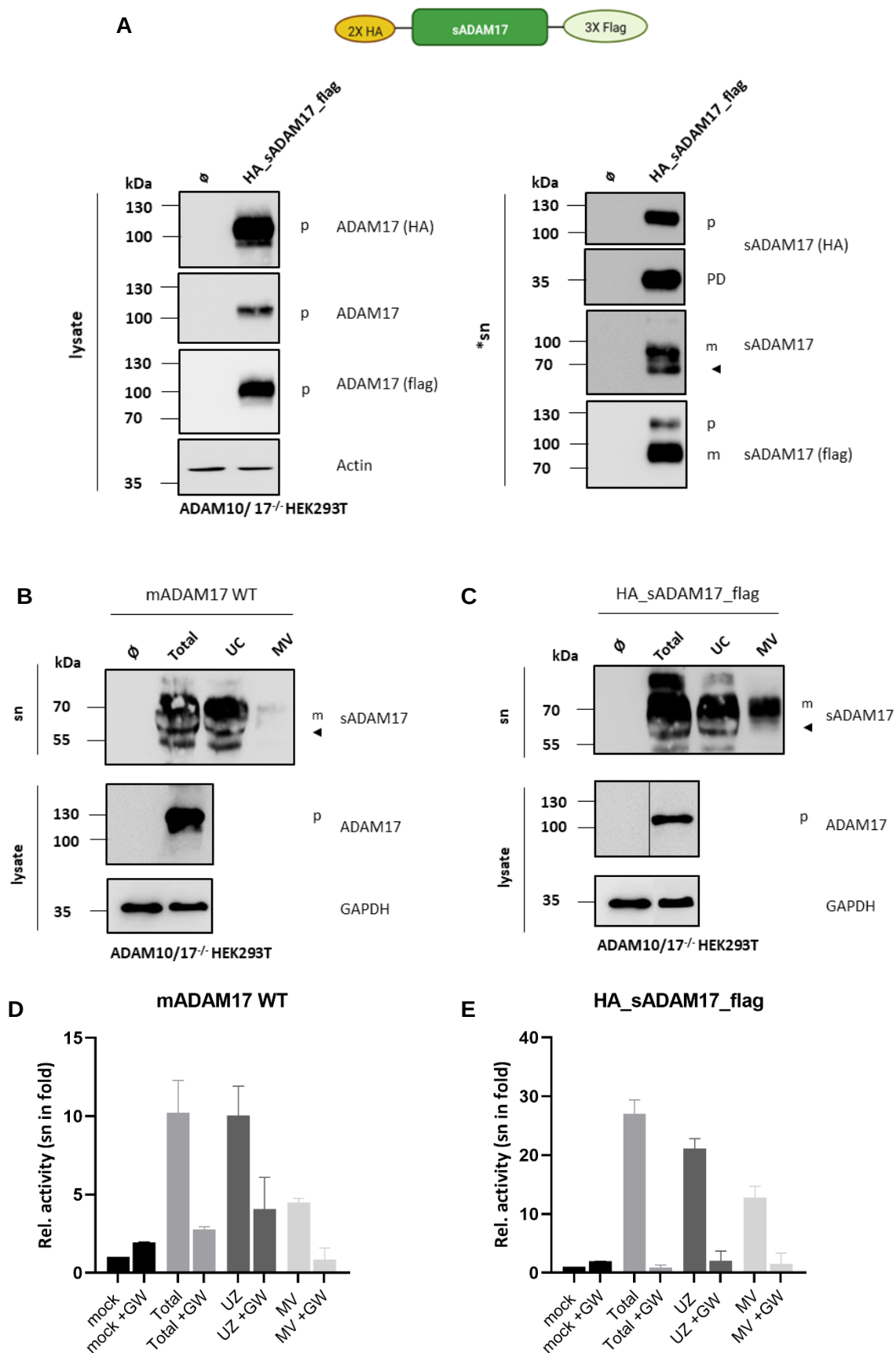


Figure 18: Biochemical characterization of soluble ADAM17

To characterize the ectodomain of ADAM17, overexpressed soluble ADAM17 with an N-terminal HA tag and C-terminal Flag tag was generated via PCR. The plasmid was expressed in ADAM10/17^{-/-} HEK 293T cells. (A)

Results

Western blot analysis of ADAM10/17^{-/-} HEK 293T cells transfected with HA_sADAM17_flag. Anti-HA-tag antibody detected the pro-form (p) and pro-domain (PD) in the ultra-centrifuged and TCA precipitated supernatant (*sn). Anti-flag antibodies detected the pro (p) and the mature form (m) of sADAM17. Anti-ADAM17 antibody detected the mature and further processed form (black arrowhead) of sADAM17. Anti-flag antibodies detected the pro (p) and the mature (m) form of sADAM17. Anti-ADAM17 antibody detected the mature and further processed form of sADAM17. (B, C) Comparison via western blot for the expression profile and release of soluble ADAM17 from the cells transfected with overexpressing mADAM17 WT and HA_sADAM17_flag. (D, E) Relative activity of proteolytically released sADAM17 and overexpressed sADAM17 against TNF- α based fluorogenic peptide in ultra-centrifuged supernatant. The activity was inhibited in the presence of GW (3 μ M). n=2 technical replicates; Mean $\pm$ SD, for two replicates.

In parallel, ADAM10/17^{-/-} HEK293T cells transfected with active and inactive murine constructs for sADAM17 and membrane-bound ADAM17 were treated with biotin for cell surface biotinylation to assess if the generated ectodomain of ADAM17 is released from the cell surface. In the cell surface biotinylation experiment, mature ADAM17 was only observed in the cells transfected with active and inactive membrane-bound mADAM17. No remnant form of the sADAM17 was detected, implying that the ectodomain is well-secreted into the extracellular space (Figure 19).

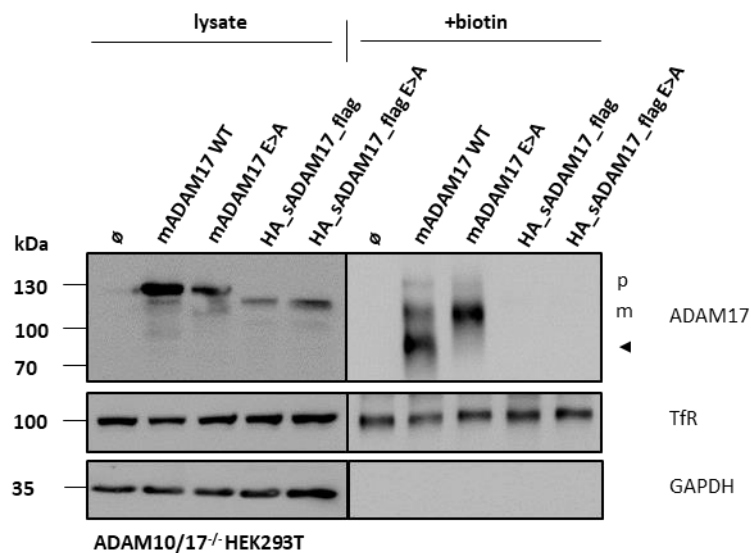


Figure 19: Cell surface biotinylation of mADAM17 variants

To check whether the overexpressed HA_sADAM17_flag is well secreted, and no remnant form of the protease remains on the cell surface, a biotinylation assay was performed on ADAM10/17^{-/-} HEK 293T cells transfected with active and inactive variants of membrane-bound and soluble mADAM17. Transferrin (Tfr) and GAPDH served as biotinylation and loading control, respectively. No cell surface presence of the overexpressed truncated ectodomain was observed. The pro, mature, and mature processed forms of ADAM17 are marked with p, m, and the black arrowhead* respectively.

Membrane-bound ADAM17 has been shown to carry N-glycosylations in the extracellular part, including the pro-domain (Gooz, 2010; Peiretti et al., 2003). Further studies showed that glycosylation is an important post-translational modification that directly affects the activity and inhibition of ADAM17 (Chavaroche et al., 2014). Therefore, it was of interest to check whether the soluble ADAM17 is glycosylated like its membrane-bound counterpart. The glycosylation pattern of soluble ADAM17 was studied by deglycosylation of overexpressed sADAM17 and proteolytically released ADAM17 in the supernatant and the lysates for the overexpressed sADAM17 and membrane-bound ADAM17. The cell lysates and concanavalin-A enriched supernatants were treated with either N-glycosidase F (PNGase F or PF) and Endoglycosidase-H (Endo H or EH) and probed via immunoblotting. PNGase F is known to remove all N-glycosylation which indicates that the protein has attained its glycans in the median Golgi. On the other hand, Endo H processes only high mannose-type glycans added in the endoplasmic reticulum (ER) (Trimble & Tarentino, 1991). The application of glycosidases helps in determining the localization and trafficking of the target proteins.

For both the constructs, in the supernatant, sADAM17 only gets deglycosylated by PNGase F, which suggests that the sADAM17 is fully glycosylated and properly folded (Figure 20A). In the lysates from ADAM17 WT, the pro (p) and the mature (m) form seemed to migrate to ~100 kDa upon treating with Endo H, however, the mature form migrated further to 75 kDa upon treatment with PNGase F. On the contrary, for the HA_sADAM17_flag, deglycosylation of pro and mature forms occurs but the bands migrate to the same distance after treatment with both the enzymes (Figure 20A). Additionally, the deglycosylation performed in iRhom1/2^{-/-} HEK 293T cells also revealed a similar profile for soluble ADAM17 in the supernatant (Figure 20B). In the case of mADAM17 WT, no mature form was detected when iRhoms were absent, but in the case of HA_sADAM17_flag, the pro and mature forms were detected in the lysates. The release of soluble ADAM17 with a similar glycosylation profile in both approaches, once again suggests the maturation and trafficking of sADAM17 independent of iRhoms.

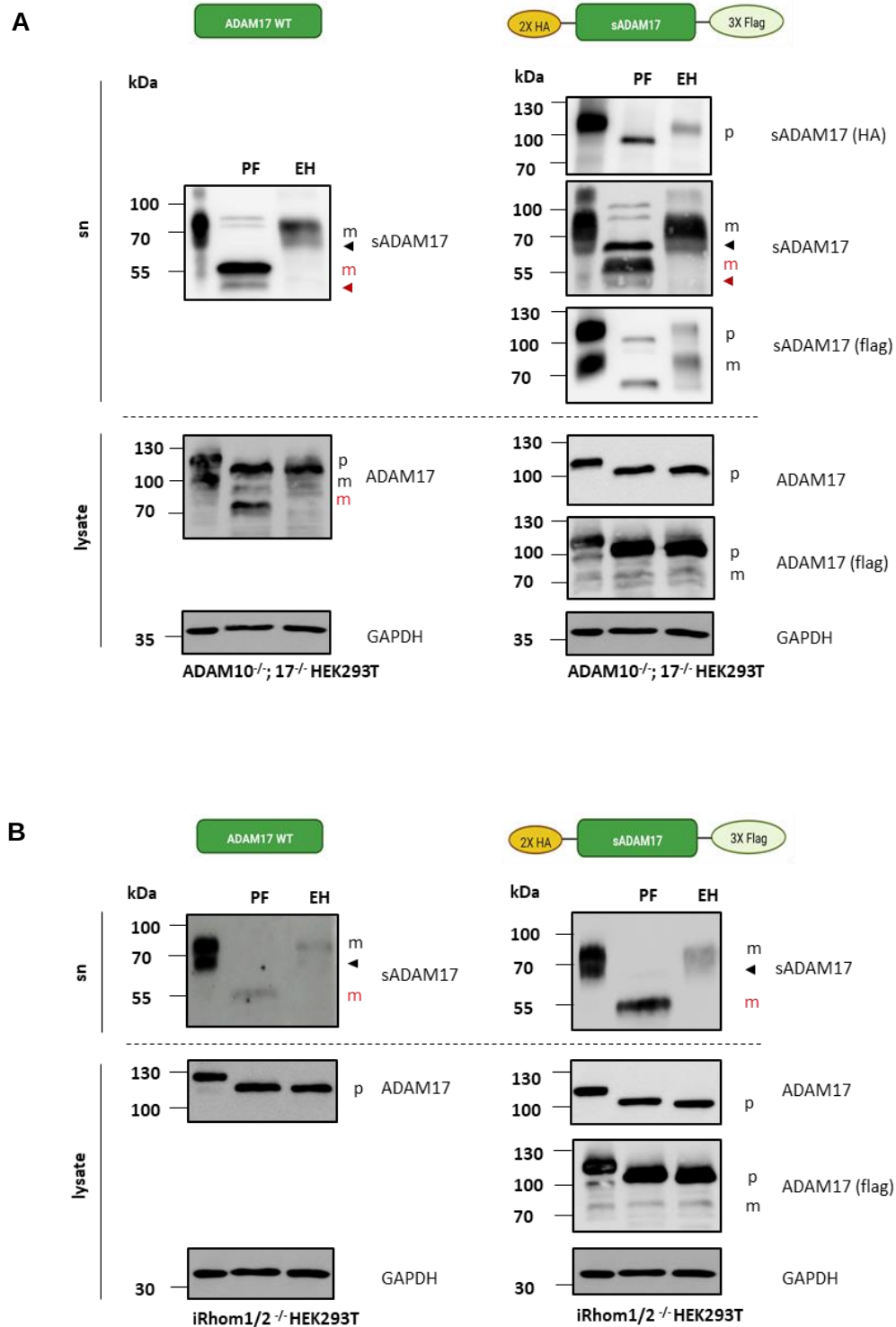


Figure 20: Soluble ADAM17 is a complex glycosylated protease

Glycosylation pattern of murine soluble ADAM17 was determined in (A) ADAM10/17^{-/-}, (B) iRhomb1/2^{-/-} HEK293T cells. The cells were transfected with mADAM17 WT and sADAM17 overexpression (HA_sADAM17_flag) constructs. Following 24h starvation in serum-free media, the supernatant samples were concentrated, and concanavalin A precipitated. Then the supernatants and lysates were subjected to deglycosylation with PNGase F (PF) and Endo H (EH). (A, B) On the left panel, soluble ADAM17 was generated by the shedding of mADAM17 WT compared with the overexpressed HA_sADAM17_flag on the right. The pro-form, mature form, and mature processed form of ADAM17 are marked by (p), (m), and black arrowhead respectively. The deglycosylated mature and processed forms of ADAM17 are shown with red markings.

3.3.2 EGFR ligand shedding by soluble ADAM17

To examine whether the proteolytic activity of sADAM17 is restricted to soluble substrates or can also function as a sheddase, the predefined substrates for membrane-bound ADAM17 were tested. The membrane-bound ADAM17 is actively involved in the EGFR signaling pathways via the ectodomain shedding of EGFR ligands like AREG, HB-EGF, TGF- α and EREG (Sunnarborg et al., 2002). Therefore, a comparative analysis between membrane-bound ADAM17 and sADAM17 (HA_sADAM17_flag) for the shedding of AP-tagged EGFR ligands was conducted. Figure 21A shows an overview of EGFR-ligands that are substrates to membrane-bound ADAM10 and ADAM17. The experimental setup is briefly shown in figure 21B. ADAM10/17^{-/-} HEK293T cells were single or co-transfected with N-terminally alkaline phosphatase tagged EGFR ligands (Sahin et al., 2004) and mADAM17 variants (HA_sADAM17_flag, HA_sADAM17_flag E>A and mADAM17 WT). The shedding of EGFR-ligands was analyzed via western blots for the ultra-centrifuged and TCA precipitated supernatant and the alkaline phosphatase (AP) activity assay for the corresponding cleared supernatants.

Each western blot is representative of three independent experiments and the relative AP activity is calculated by normalizing the absorbance for ligand-only supernatant for three independent experiments. The overexpressed sADAM17 (HA_sADAM17_flag) was able to release soluble AREG, and EREG at similar intensity as compared to mADAM17 WT (Figure 21C, E). As expected, membrane-bound ADAM17 did not shed BTC, but interestingly, soluble BTC was detected in the supernatants upon co-transfection with sADAM17 (Figure 21D). The respective relative AP activity analyzed in the supernatant concurs with the western blot profile of sAREG, sBTC, and sEREG (Figure 22A, B, C). sTGF- α was increased in the presence of membrane-bound ADAM17, and some noticeable amounts were also detected in the presence of overexpressed sADAM17 (Figure 21F). However, a significant relative AP activity was only achieved by sTGF- α generated by membrane-bound ADAM17 (Figure 22D). In the case of HB-EGF, a known substrate of membrane-bound ADAM17, released sHB-EGF was only detected when co-transfected with mADAM17 WT (Figure 21G), and the AP-activity analysis resembles the western blot profile (Figure 22E). Figure 22F gives an overview of potential shared and individual substrates of membrane-bound ADAM17 and overexpressed ADAM17.

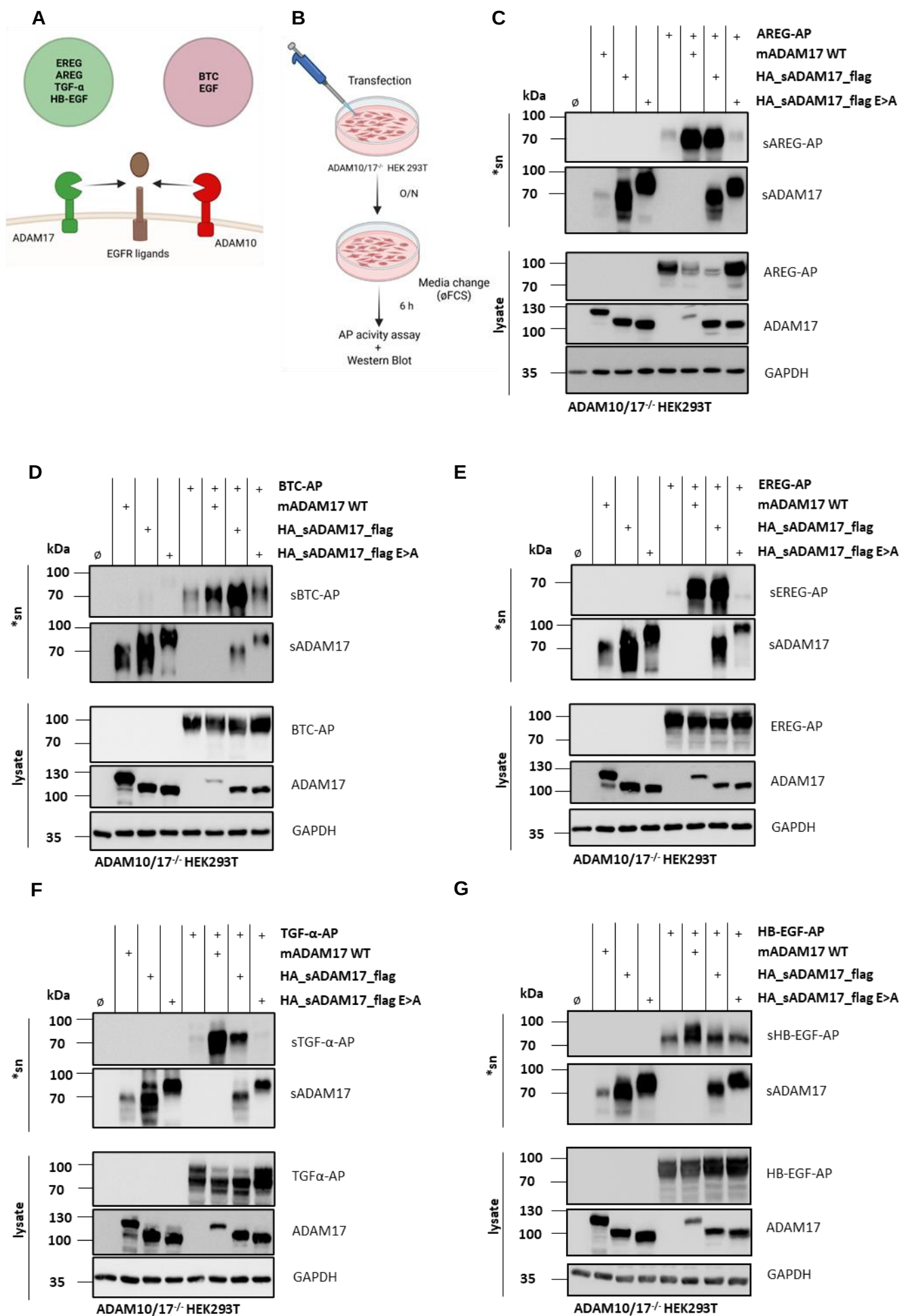
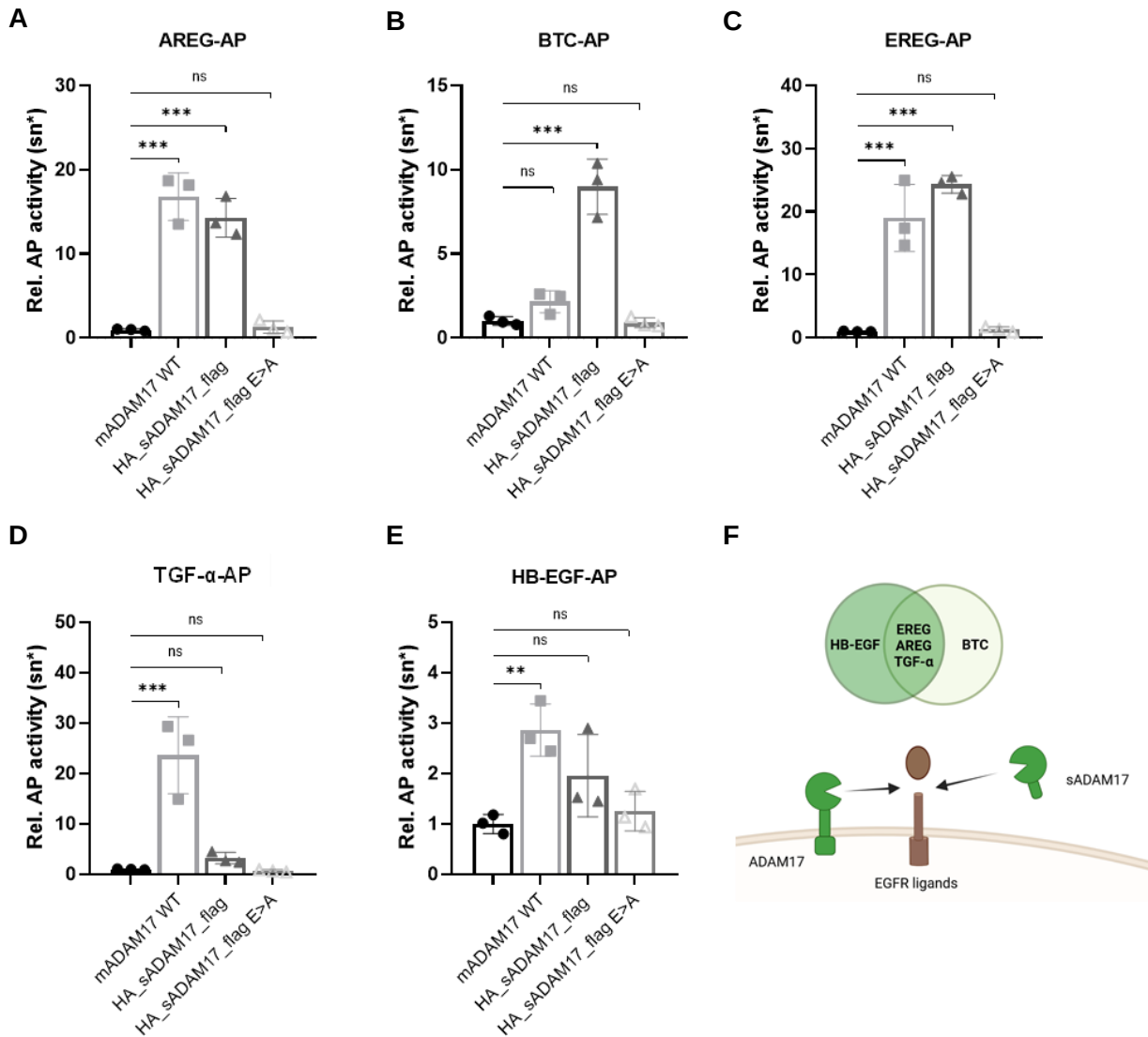


Figure 21: sADAM17 is capable of shedding different EGFR ligands in *cis*

(A) Cartoon showing the known EGFR ligand as substrates for membrane-bound ADAM17 (in green) and ADAM10 (in red). (B) Experimental scheme. (C-G) Representative western blots (n=3), for analysis of shedding of AP-tagged EGFR ligands by mADAM17 variants; mADAM17 WT, HA_sADAM17_flag, and HA_sADAM17_flag E>A. (C) AREG-AP, (D) BTC-AP, (E) EREG-AP, (F) TGF- α -AP, (G) HB-EGF-AP. The EGFR-AP ligands were detected by the antibody against the AP tag, ADAM17 was detected by an antibody against its ectodomain and GAPDH was used as a loading control.

(*sn): supernatants were ultracentrifuged and TCA-precipitated.

**Figure 22: Alkaline-phosphatase activity by sEGFR ligands released in *cis***

(A-E) Relative alkaline phosphatase (AP) activity analysis in ultracentrifuged supernatant for soluble EGFR-AP ligands generated by mADAM17 variants; mADAM17 WT, HA_sADAM17_flag, and HA_sADAM17_flag E>A. Relative AP activity is analyzed via graph pad prism (n=3), data represented as mean $\pm$ SD and statistical analysis was performed by one-way ANOVA followed by Dunnett's post hoc test (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

(F) an overview of potential shared and individual substrates of membrane-bound ADAM17 and overexpressed ADAM17.

3.3.3 EGFR ligand shedding by proteolytically released soluble ADAM17

The overexpressed sADAM17 (HA_sADAM17_flag) was able to release soluble EGFR ligands like AREG and EREG in a comparable manner as membrane-bound ADAM17 (mADAM17 WT). Additionally, significant shedding of BTC was also observed in the presence of sADAM17. Now, to assess whether the proteolytically released ADAM17 could also shed the EGFR ligands in *trans*, ADAM10/17^{-/-} HEK293T cells were transfected with mADAM17 WT, and the conditioned media were transferred to the cells transfected with different EGFR ligands. The cells were starved in serum-free media and the conditioned media enriched with proteolytically released soluble ADAM17 was collected. The conditioned media was concentrated twice and transferred to the cells transfected with EGFR-AP ligands. In parallel, to compare the shedding capacity of sADAM17 in conditioned media with membrane-bound ADAM17 the cells were co-transfected with mADAM17 and EGFR-AP ligands (Figure 23A). sADAM17 could release soluble forms of AREG, BTC, EREG, and TGF- α (Figure 23B-E). Notably, the shedding of AREG via sADAM17 in *cis* and *trans* differs in terms of signal intensities as compared to membrane-bound ADAM17 (Figure 23B). This suggests that the shedding of AREG already occurs within the secretory pathway when sADAM17 is co-expressed with the ligand. The shedding of HB-EGF was again only observed in the presence of membrane-bound ADAM17 (Figure 23F). Further, the AP-activity assay was performed to check for the released sEGFR-AP ligands in the ultracentrifuged supernatant samples. The activity profile coincided with the western blot signals for the EGFR ligands. significant relative AP-activity was obtained with sAREG, sEREG sTGF- α , and sHB-EGF in the presence of membrane-bound ADAM17 (Figure 24A, C, D, E). On the other hand, for sAREG, sBTC, sEREG, and sTGF- α generated in *trans*, significant relative activity was observed (Figure 24A-D). Taken together, AREG, BTC, EREG, and TGF- α once again proved to be the substrates of sADAM17 *in vitro* (Figure 24F).

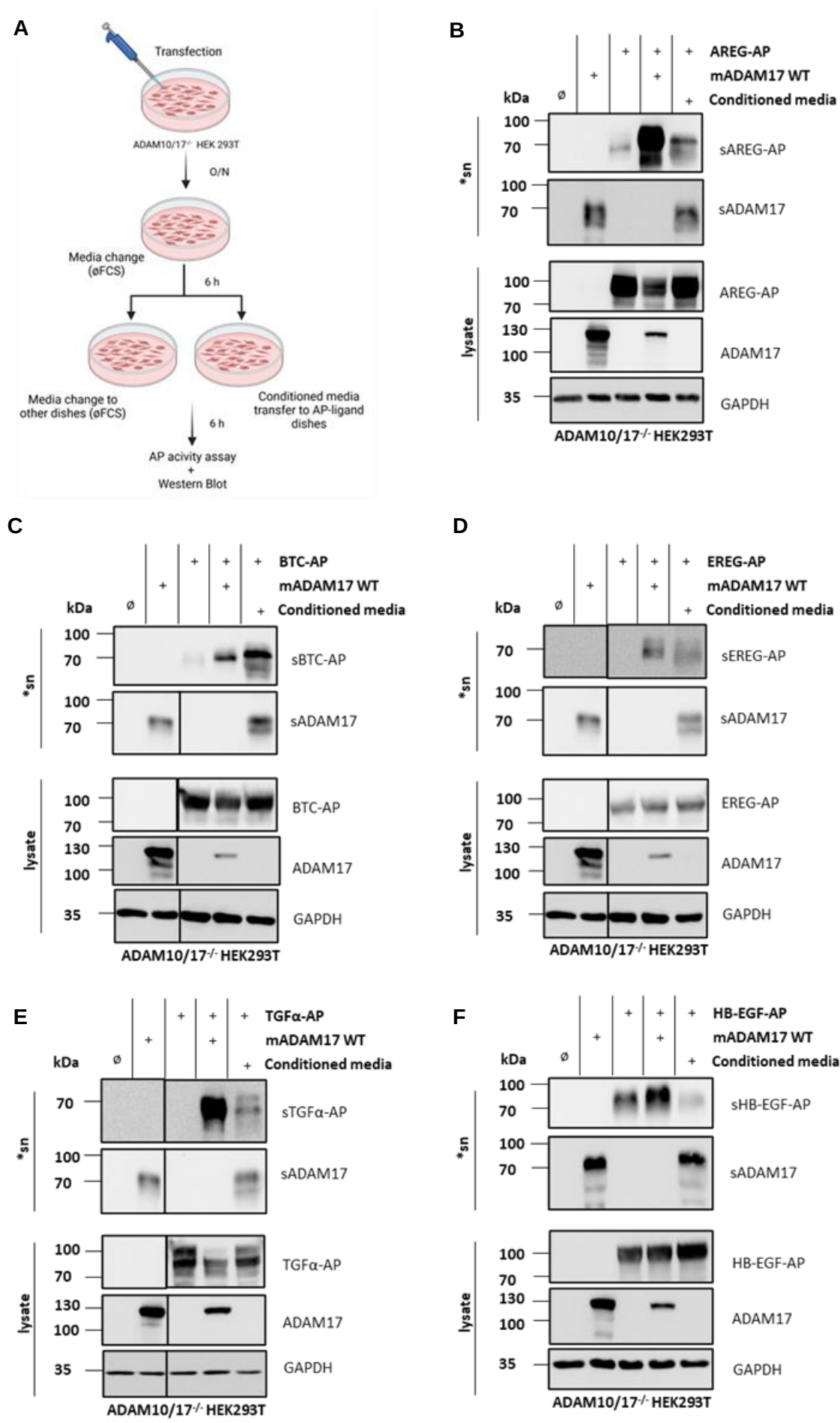
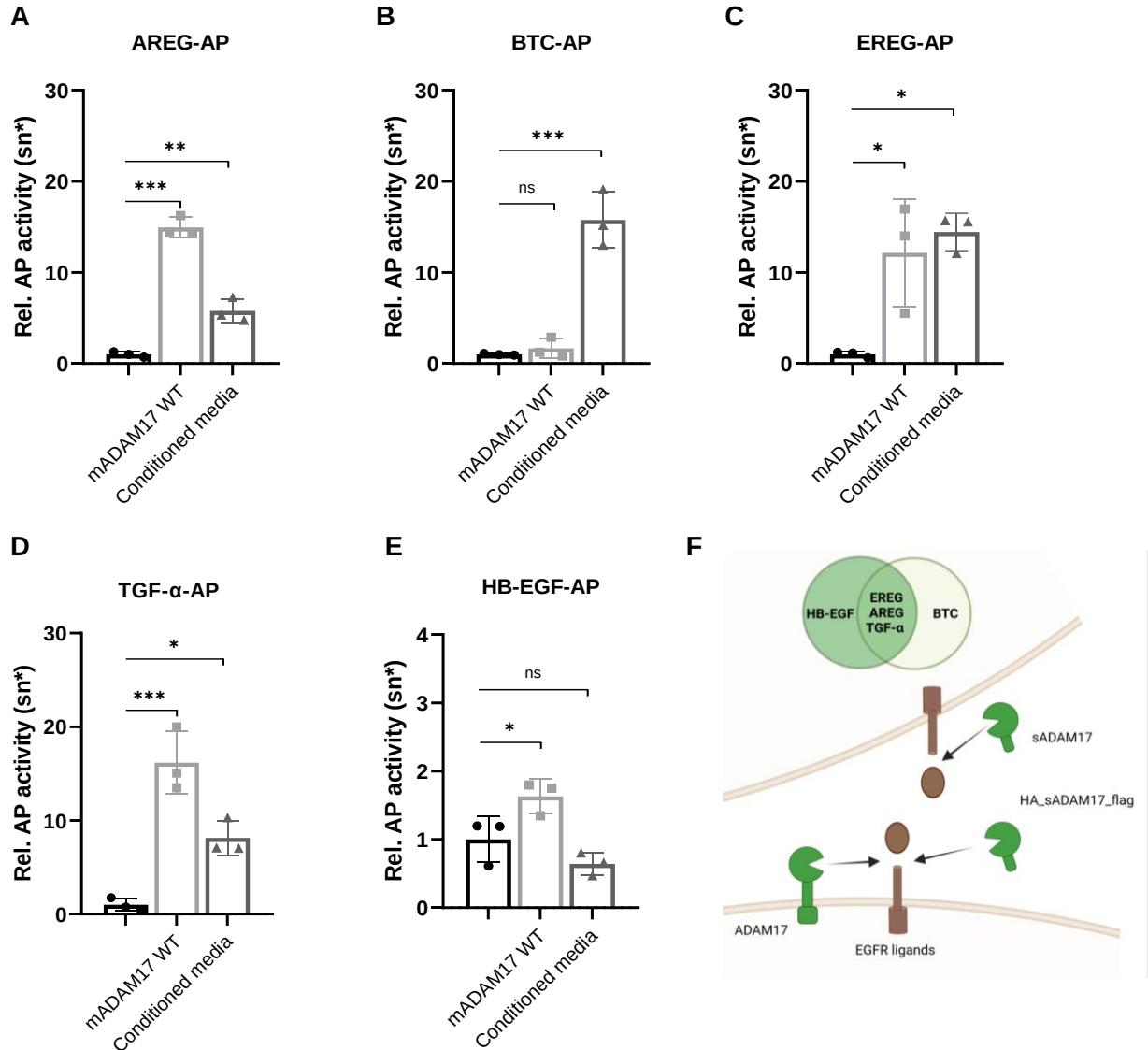


Figure 23: sADAM17 is capable of shedding different EGFR ligands in *trans*

(A-E) Western blots analysis of shedding of AP-tagged EGFR ligands by mADAM17 WT and sADAM17 in conditioned media; (A) AREG-AP, (B) BTC-AP, (C) EREG-AP, (D) TGF- α -AP, (E) HB-EGF-AP. The EGFR-AP ligands were detected by the antibody against the AP tag, ADAM17 was detected by the antibody against its ectodomain and GAPDH was used as a loading control.

(*sn): supernatants were ultracentrifuged and TCA-precipitated.

**Figure 24: Comparative alkaline-phosphatase activity by sEGFR ligands released in *cis* and *trans***

(A-E) Relative alkaline phosphatase (AP) activity analysis in ultracentrifuged supernatant for soluble EGFR-AP ligands generated by mADAM17 WT; and sADAM17 in conditioned media. Relative AP activity is analyzed via graph pad prism (n=3), data represented as mean $\pm$ SD and statistical analysis was performed by one-way ANOVA followed by Dunnett's post hoc test (*: p<0.05, **: p<0.01; ***: p<0.001). (F) Based on the data collected from western blots and AP-activity assay, AREG, BTC, EREG and TGF- α proved to be the substrates of sADAM17 *in vitro*.

3.3.4 EGFR ligand shedding in the absence of iRhom1/2

iRhom1/2 are proven to be crucial in the ADAM17-mediated EGFR signaling via shedding of amphiregulin, HB-EGF, epiregulin, and TGF- α . In the absence of both the iRhoms, no constitutive or PMA-induced shedding of these ligands was observed (Li et al., 2015). However, as shown in previous sections, if the shedding of ADAM17 and the maturation of sADAM17 can occur independent of iRhom1/2, sADAM17 can still shed EGFR ligands in an iRhom-independent way. Therefore, the shedding capacity of sADAM17 in iRhom1/2^{-/-} HEK293T cells was analyzed. Since AREG was significantly shed in *cis* and *trans* by sADAM17, the cells were single or co-transfected with AREG-AP and mADAM17 variants. The setup for the shedding experiment was the same as the previous experiment performed in ADAM10/17^{-/-} HEK293T cells. Intriguingly, in the absence of iRhom1/2, the shedding of AREG was observed by membrane-bound ADAM17 and by overexpressing sADAM17 (HA_sADAM17_flag) (Figure 25A). As expected, no shedding was observed with HA_sADAM17_flag E>A. The relative AP activity data also revealed a similar pattern, where significant AP activity was observed in the presence of membrane-bound ADAM17 and HA_sADAM17_flag (Figure 25C). In the experimental setup with the conditioned media, to attain comparable levels of sADAM17 in the conditioned media as overexpressed sADAM17, the conditioned media was concentrated 2 times with Amicon Ultra concentrator tubes. Here, the major shedding of AREG was observed in the presence of membrane-bound ADAM17, and a faded band was also observed in the supernatant cells supplied with conditioned media (Figure 25B), but significant activity was only obtained with mADAM17 WT (Figure 25D). Even though a significantly high AP activity was not achieved, as compared to mock-transfected cells, a slightly higher activity trend was noted (Figure 25D).

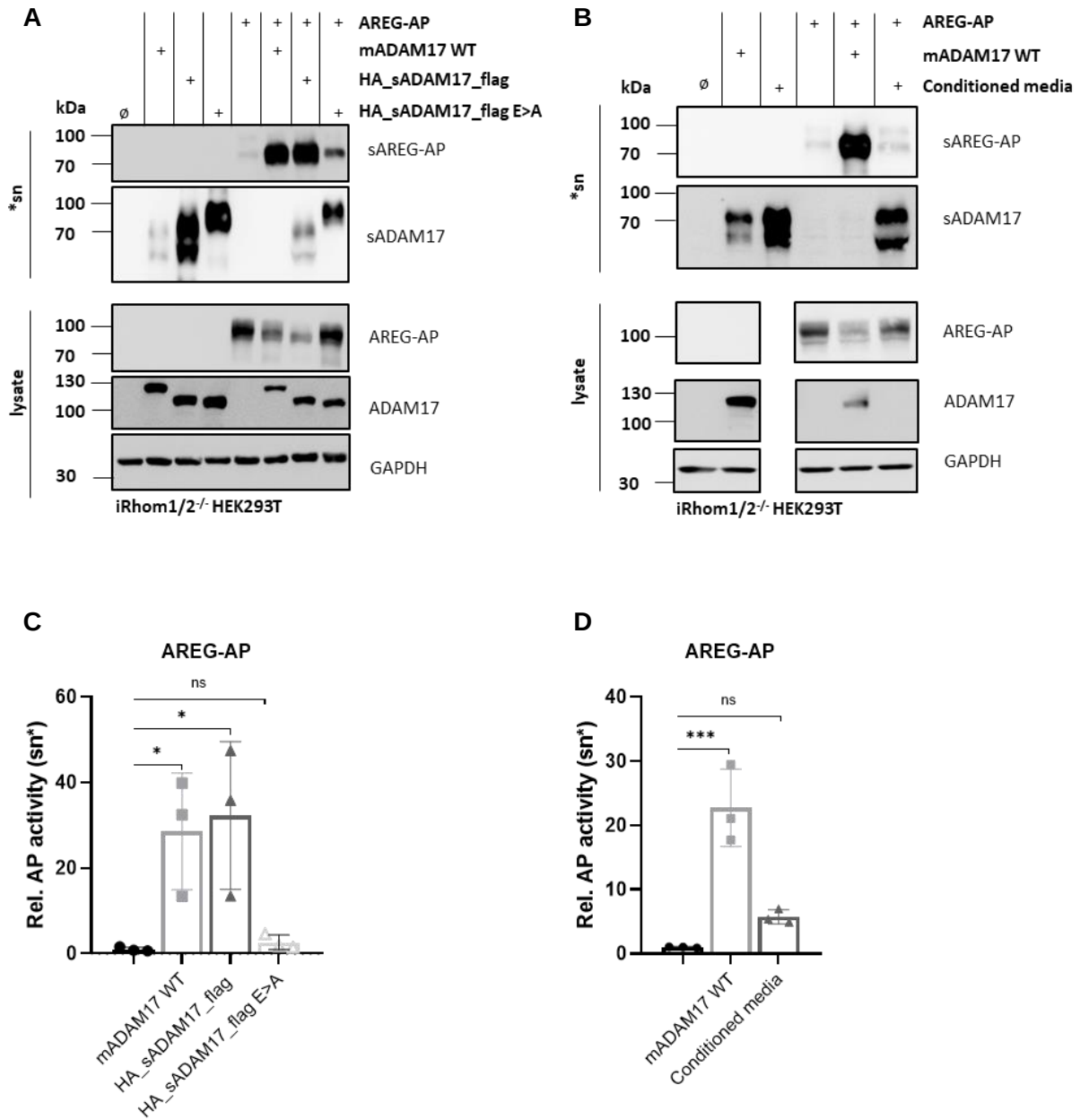


Figure 25: sADAM17mediated AREG-AP shedding in absence of iRhom1/2

Representative western blots (n=3) for shedding analysis of AREG-AP via mADAM17 variants; mADAM17 WT, HA_sADAM17_flag, and HA_sADAM17_flag E>A in iRhom1/2^{-/-} HEK 293T cells (A) and via in conditioned media enriched with proteolytically released sADAM17 (B) in iRhom1/2^{-/-} HEK 293T cells. (C, D) Relative AP activity is presented in the lower panel.

Relative AP activity is analyzed via graph pad prism (n=3), data represented as mean $\pm$ SD, and statistical analysis was performed by one-way ANOVA followed by Dunnett's posthoc test (*: p<0.05, **: p<0.01; ***: p<0.001).

In ADAM10/17^{-/-} HEK293T cells, the shedding of HB-EGF was observed only in the presence of membrane-bound ADAM17 and sADAM17 was unable to shed HB-EGF in *cis* and *trans*. Therefore, HB-EGF was single or co-transfected with mADAM17 variants as a negative control for shedding via sADAM17. Surprisingly in the absence of iRhom1/2, HB-EGF was observed to be shed by membrane-bound and sADAM17 (Figure 26A). A trend of increase in relative AP activity was also noted however, the data did not show any significance (Figure 26C).

Next, to analyze the shedding of HB-EGF in *trans*, the conditioned media was supplied to the cells. The AP activity and shedding were examined in the presence of membrane-bound ADAM17 and sADAM17 in conditioned media via western blot and AP activity assay (Figure 26B, D). Interestingly, for HB-EGF the shedding was considerably high with the conditioned media enriched with sADAM17 in the WT and iRhom1/2^{-/-} cells (Figure 26B), a similar trend was observed for the AP activity assay (Figure 26D). The depletion in the activity of membrane-bound ADAM17 over time might be a result of autocatalysis or time-dependent degradation of ADAM17. In sum, depending upon the substrate accessibility, shedding via sADAM17 could occur in the secretory pathway or on the cell surface.

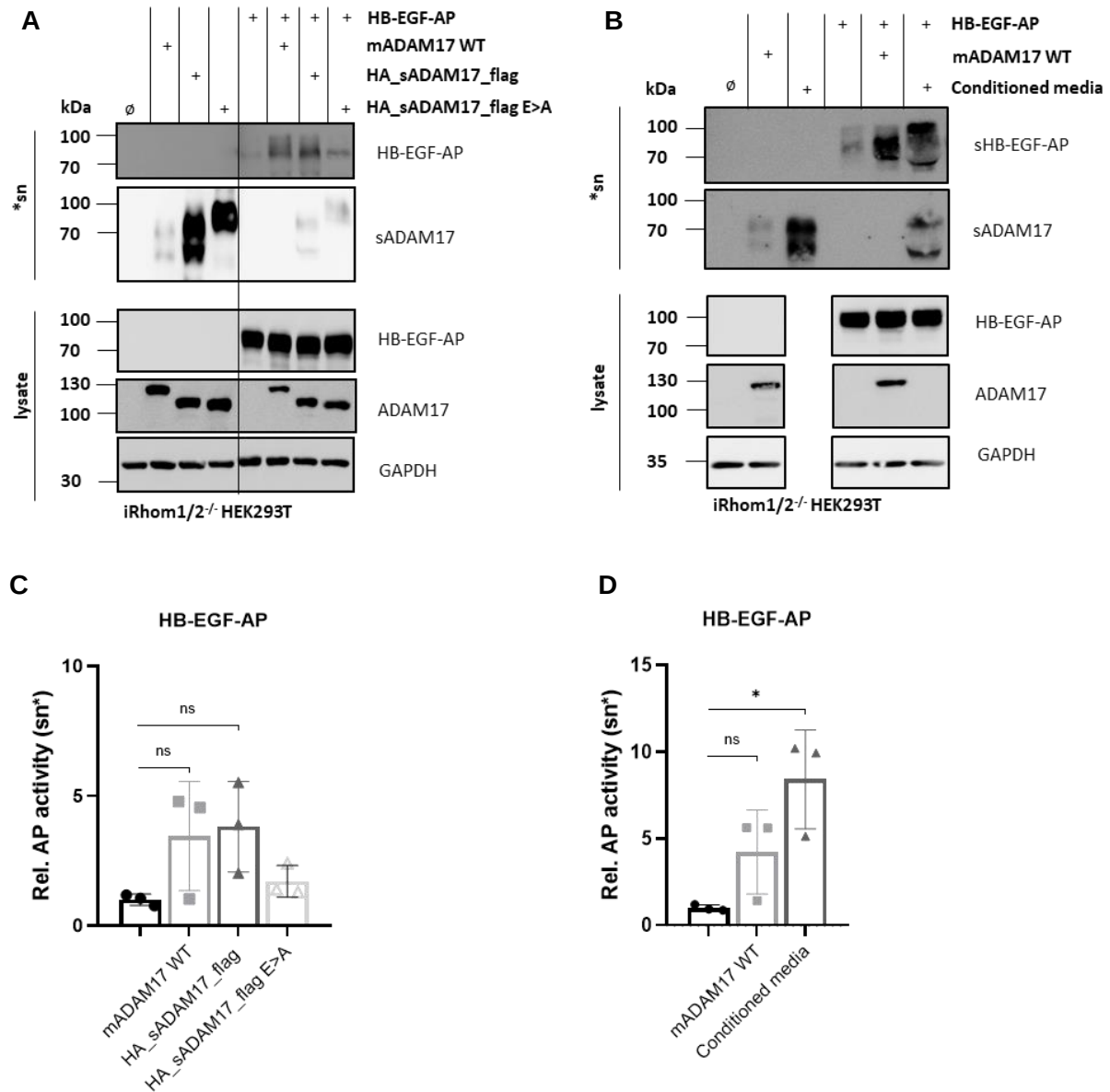


Figure 26: sADAM17-mediated HB-EGF-AP shedding in the absence of iRhom1/2

Representative western blots (n=3) for shedding analysis of HB-EGF-AP via mADAM17 variants; mADAM17 WT, HA_sADAM17_flag, and HA_sADAM17_flag E>A in iRhom1/2^{-/-} HEK 293T cells (A) and via in conditioned media enriched with proteolytically released sADAM17 (B) in iRhom1/2^{-/-} HEK 293T cells. (C, D) Relative AP activity is presented in the lower panel.

#Relative AP activity is analyzed via graph pad prism (n=3), data represented as mean $\pm$ SD and statistical analysis was performed by one-way ANOVA followed by Dunnett's post hoc test (*: p<0.05, **: p<0.01; ***: p<0.001).

3.4 Identification of putative new substrates of ADAM8 and ADAM17 in breast cancer cells

The secretome from the 3D culture of MDA-MB-231 cells has previously been analyzed for the abundance of proteins that could be potential serum markers for the prognosis and diagnosis of TNBC (Ziegler et al., 2016). In the same study, various ECM proteins, growth factors, proteases, and cytokines that could contribute to metastasis and proliferation seem to be highly abundant (Ziegler et al., 2016). This suggested that multiple proteins play a role in tumor microenvironment and contribute to malignancies. As mentioned earlier in TNBC, proliferation, metastasis and invasion could be a result of activities from ADAM8 (Conrad et al., 2018; Romagnoli et al., 2014) and ADAM17 (Giricz et al., 2013; McGowan et al., 2013; Zheng et al., 2009). Therefore, it was interesting to screen for the peptides generated by possible cleavage events caused by ADAM8 or ADAM17 in the secretome of MDA-MB-231 cell variants. For the identification of novel substrates of endogenous ADAM8 and ADAM17, the conditioned media of WT, hA8KO and hA8EQ MDA-MB-231 cells were analyzed via the N-terminomics TAILS approach. A scheme for the substrate screening is represented in figure 27. As described previously in section 2, the cells were grown to confluency and were then starved in serum-free media. Thereafter, the conditioned media is collected and concentrated for the analysis of proteolytically generated peptides in the secretome of the cells. From each sample, 3 technical replicates were subjected to a TMT-multiplexing. After the enrichment of N-terminally tagged peptides, the samples were analyzed via LC-MS/MS.

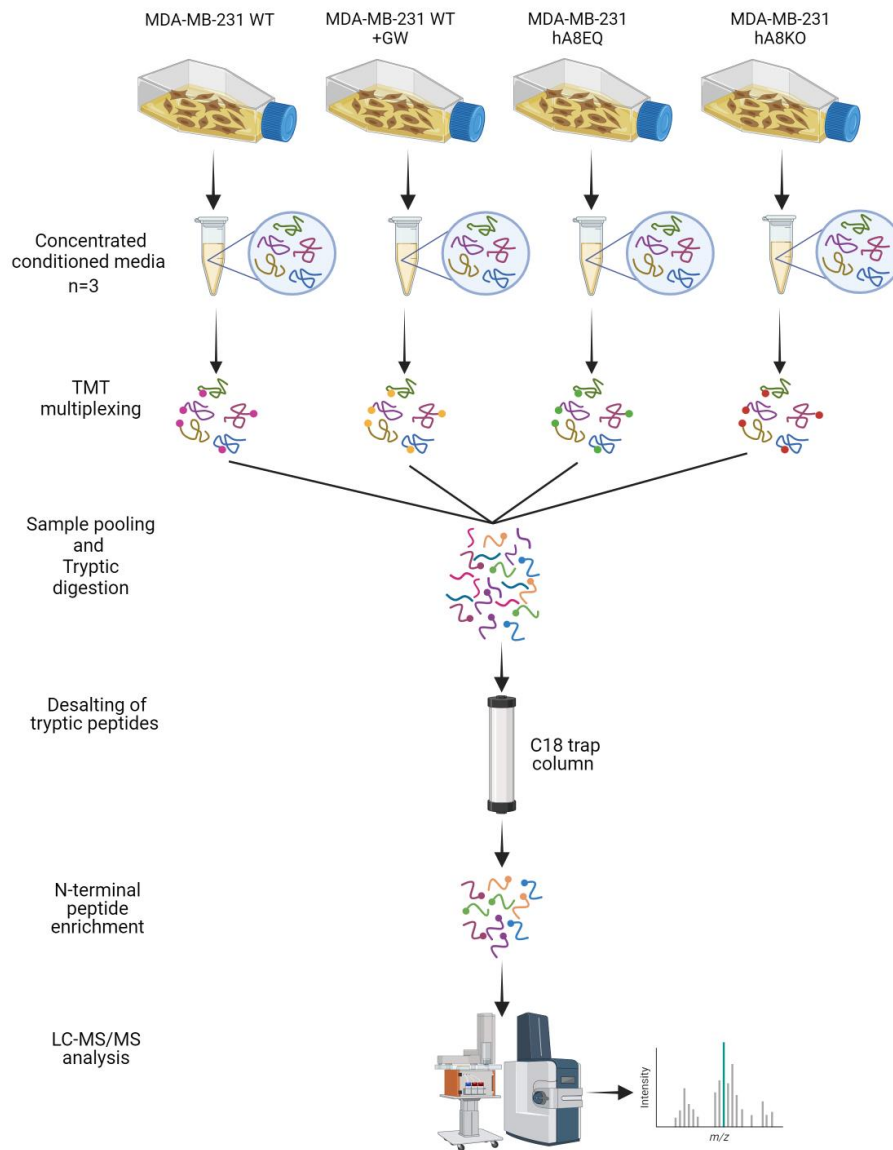


Figure 27: N-terminomics TAILS analysis scheme

For the identification of new substrates in breast cancer cells, MDA-MB-231 cell variants were analyzed. Secretome from wildtype (WT), wildtype+3 μ M GW (WT+GW), human ADAM8 knockout (hA8KO), and human ADAM8 E335Q (hA8E>Q) cells were harvested and concentrated. 3 technical replicates from each sample were subjected to TMT-labelling and tryptic digestion. Further, the N-terminally labeled peptides were enriched and subjected to analysis via LC-MS/MS.

A total of 1236 N-terminally TMT tagged, highly confident peptides were quantified that belonged to 555 unique proteins. The TAILS-identified unique peptides are shown in volcano plots where the cleavage events in WT MDA-MB-231 cells are compared to when ADAM8 is absent (hA8 KO) or inactivated (hA8 E>Q) and when ADAM17 is inhibited (WT+GW) (Figure 28A-C). The cleavage events for each condition were considered significant with a fold change value greater than 3 ($\log_2$ fold change ≥ 1.585 or ≤ -1.585) with the p-value ≤ 0.01 ($\log_{10}$ _Ttest = 2). The extracellular peptides are highlighted with a darker red or blue fill. The interesting

peptides are labeled with the cleavage site. Several interesting peptides like HLA class I histocompatibility antigen, an alpha chain (HLA-A or MHC I), Latent-transforming growth factor beta-binding protein 1 (LTBP1), Thrombospondin 1 (THBS1), Calreticulin (CALR) and Insulin-like growth factor-binding protein 3 (IGFBP3) appeared to be upregulated in WT cells against the cells with hA8 KO and when ADAM8 is inactive (hA8 E>Q). In HLA-A, cleavage within the alpha chain at R|F60 was identified, close to the peptide binding complex. For peptide binding, HLA-A interacts with calreticulin via a peptide-loading complex (Rizvi et al., 2011). Interestingly, two consecutive cleavage sites were identified within the C-terminal domain at M|K 358 and K|D359. The C-terminal domain of calreticulin is reported to be important for its calcium-dependent interaction and signaling pathways (Nakamura et al., 2001). Besides the known cleavage of THBS1 via BMP1 (Anastasi et al., 2020), new cleavage at H|N841 within the calcium-binding domain was also identified. Additionally, a C-terminal domain cleavage was also identified in IGFBP3. The upregulation of these peptides in the WT cells against hA8 KO and hA8 E>Q suggests that these proteins could be potential substrates to ADAM8.

As expected, not many different cleavage peptides were identified when hA8 E>Q cells were compared to hA8 KO cells (Figure 28D). However, for matrix metalloprotease-1 (MMP1), several cleavages within the catalytic domain at L|A249, D|D252, and I|D254 were found to be significantly high in the hA8 E>Q cells. In addition to that, transglutaminase-2 (TGM2), known to be involved in cancer through ECM remodeling, (Grigoriev et al., 2001; Sima et al., 2022) also seemed to be cleaved within the catalytic domain 14 amino acids before the catalytic triad at M|S253. However, this cleavage was only significant in WT and hA8 E>Q cells against hA8 KO cells. Interestingly, some GW-sensitive cleavage events such as ADAM8 H|L139 were also found, which could be a possible substrate to ADAM17.

Figure 28E shows the heatmap consisting top 35 highest and least abundant extracellular peptides generated by proteolytic cleavage in the conditioned media of WT cells as compared to hA8 KO and hA8 E>Q and (WT+GW). Depending upon the proteolytic activity, the potential new substrates of ADAM8 are marked with purple boxes, and the potential ADAM17 substrates are marked with green asterisk. To assess the functional relevance and reactome pathway analysis, 30 significantly upregulated, extracellular peptides were analyzed via Metascape (Zhou et al., 2019). The molecular functions and Reactome analysis of upregulated proteins revealed the involvement of these proteins in the ECM development and degradation, collagen binding, growth factor binding, signaling, and roles in immune response (Figure 28F). A list of all the molecular functions and the pathways analysis is attached in the supplementary information (Supplementary table 1) Although the verification of the noted cleavages is yet to be done, these events could play important roles in tumor progression, metastasis, and invasion.

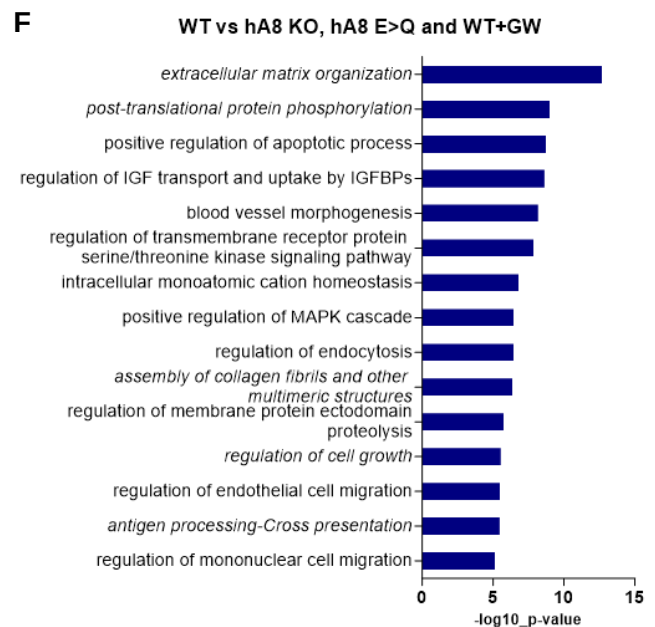
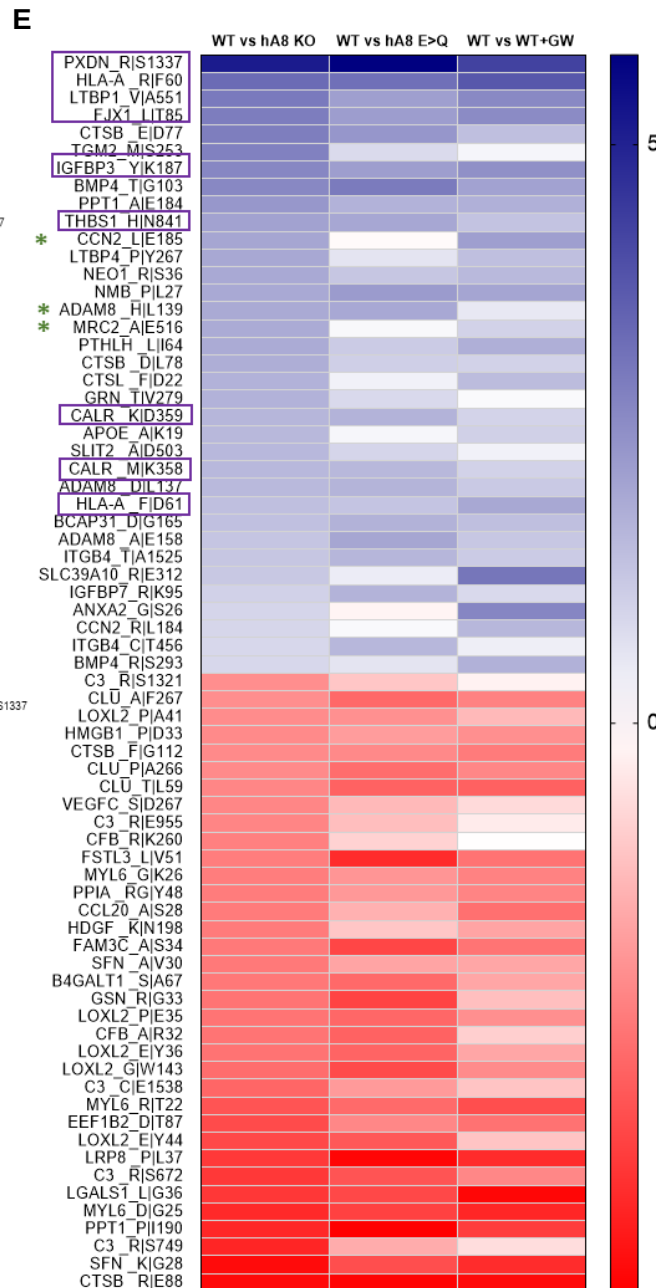
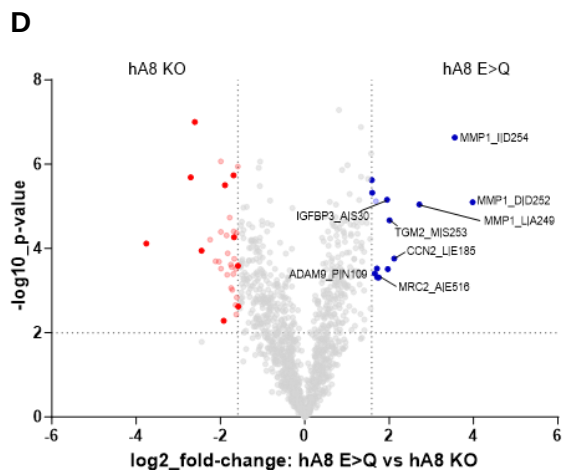
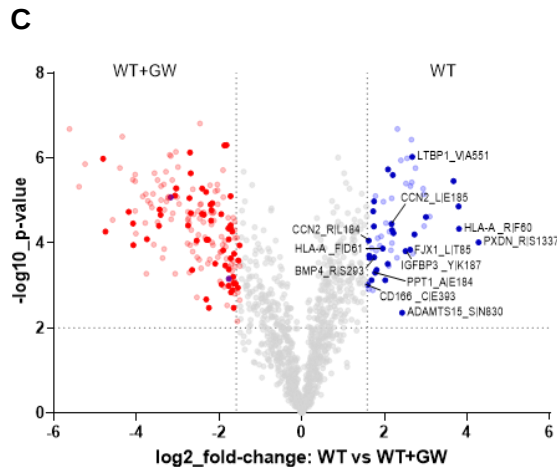
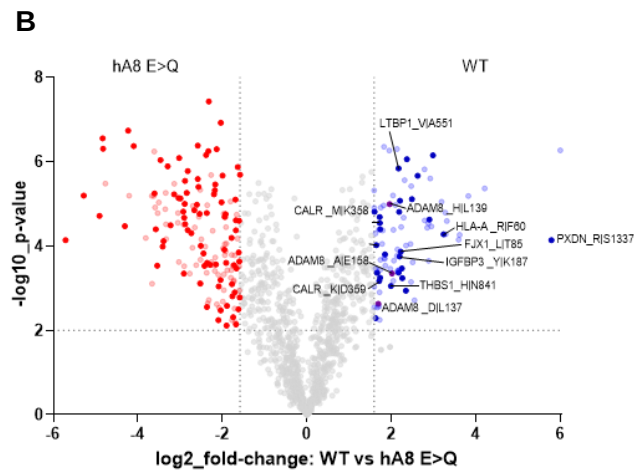
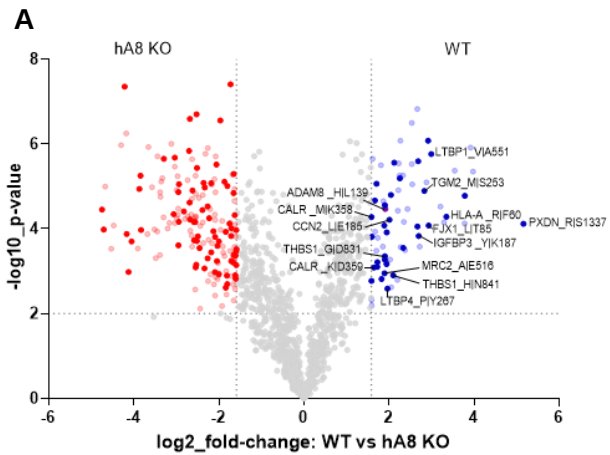


Figure 28: Novel endogenous substrate of ADAM8 and ADAM17

(A-C) Volcano plots showing all TAILS identified proteolytic events in WT MDA-MB-231 cells compared to hA8 KO, hA8 E>Q, and WT+GW. Gray dotted lines represent threshold values ($\log_2$ fold change $\geq \pm 1.585$) with the p-value = 0.01. Extracellular peptides are represented with darker blue or red filling. (D) Volcano plot showing comparison between hA8 E>Q and hA8 KO cells for all TAILS identified proteolytic events. (E) Heatmap generated for the top 35 highest (blue) and least (red) abundant peptides in the WT MDA-MB-231 cells compared to hA8KO, hA8EQ, and WT+GW. Potential new substrates for ADAM8 are marked with a purple box and for ADAM17 with green asterisk ($\log_2$ fold change ≥ 1.585 or ≤ -1.585) with the p-value ≤ 0.01 . (F) Reactome and functional enrichment analysis via Metascope for the top 30 significantly abundant peptides in WT cells in comparison to hA8 KO, hA8 E>Q, and WT+GW. The top sixteen enrichment cluster cores were plotted with the p-value. The reactome description is written in italics.

3.5 Identification of new substrates of sADAM17

In WT cells, due to the abundance of ADAM8, sADAM17 in the secretome would also be high. Therefore, for the identification of potential new substrates of sADAM17, the conditioned media (CM) with or without 3 μ M GW was transferred from the WT cells onto the hA8 KO cells. The TAILS workflow is shown in figure 29A. The secretome isolated from hA8 KO cells with the conditioned media (hA8 KO+CM) was compared to the secretome of hA8 KO+CM with GW (a hydroxamate inhibitor against ADAM17) and with hA8 KO cells. The cleavage events for each condition were considered significant with a fold change value greater than 1.5 ($\log_2$ fold change ≥ 0.585 or ≤ -0.585) with the p-value ≤ 0.01 ($\log_{10}$ _Ttest = 2). Figure 29B shows the heatmap consisting of the top 35 highest (blue) and least (red) abundant extracellular peptides generated by proteolytic cleavage in the secretome of hA8 KO+CM as compared to hA8 KO+CM+GW and hA8 KO. Depending upon the GW sensitivity and ADAM17 activity, the potential new substrates for sADAM17 are marked with a green asterisk. The identified unique peptides are shown in volcano plots where the cleavage events in hA8 KO cell +CM are compared to hA8 KO+CM+GW (Figure 29C) and when ADAM8 is absent (hA8 KO) (Figure 29D). The extracellular peptides are highlighted with darker red (low) or blue (high) fill. The interesting, upregulated peptides in the hA8 KO+CM are labeled with the cleavage site. Interestingly, in this setup, Connective tissue growth factor (CCN2 or CTGF), A disintegrin and metalloproteinase with thrombospondin motifs 15 (ADAMTS15) and C-type mannose receptor 2 (MRC2), appeared to be significantly high.

Amongst soluble proteins, a peptide belonging to ADAMTS15 with a cleavage in the spacer region at S|N830 was identified. Moreover, CCN2 is already known to be cleaved by different MMPs at the hinge region for the release of biologically active fragments (Kaasboll et al., 2018). The TAILS data identified 2 more consecutive cleavages within the hinge region at L|E185 and E|D186. Of note, MRC2 has recently been identified as an iRhom1-dependent substrate of

membrane-bound ADAM17 (Tusaus et al., 2021), however here, two cleavage sites within the ectodomain were found at G|A514 and A|E516. Out of which G|A514 was GW sensitive. Additionally, another interesting site within the propeptide of ADAM8 at H|L139 also appeared to be inhibited in the presence of GW. The molecular functions and Reactome analysis of upregulated proteins in the case of conditioned media-treated cells also revealed the involvement of these proteins in the ECM development and degradation, collagen binding, cell migration, growth factor binding, signaling, and roles in immune response (Supplementary figure 1). A list of all the molecular functions and the pathways analysis is attached in the supplementary information (Supplementary table 1).

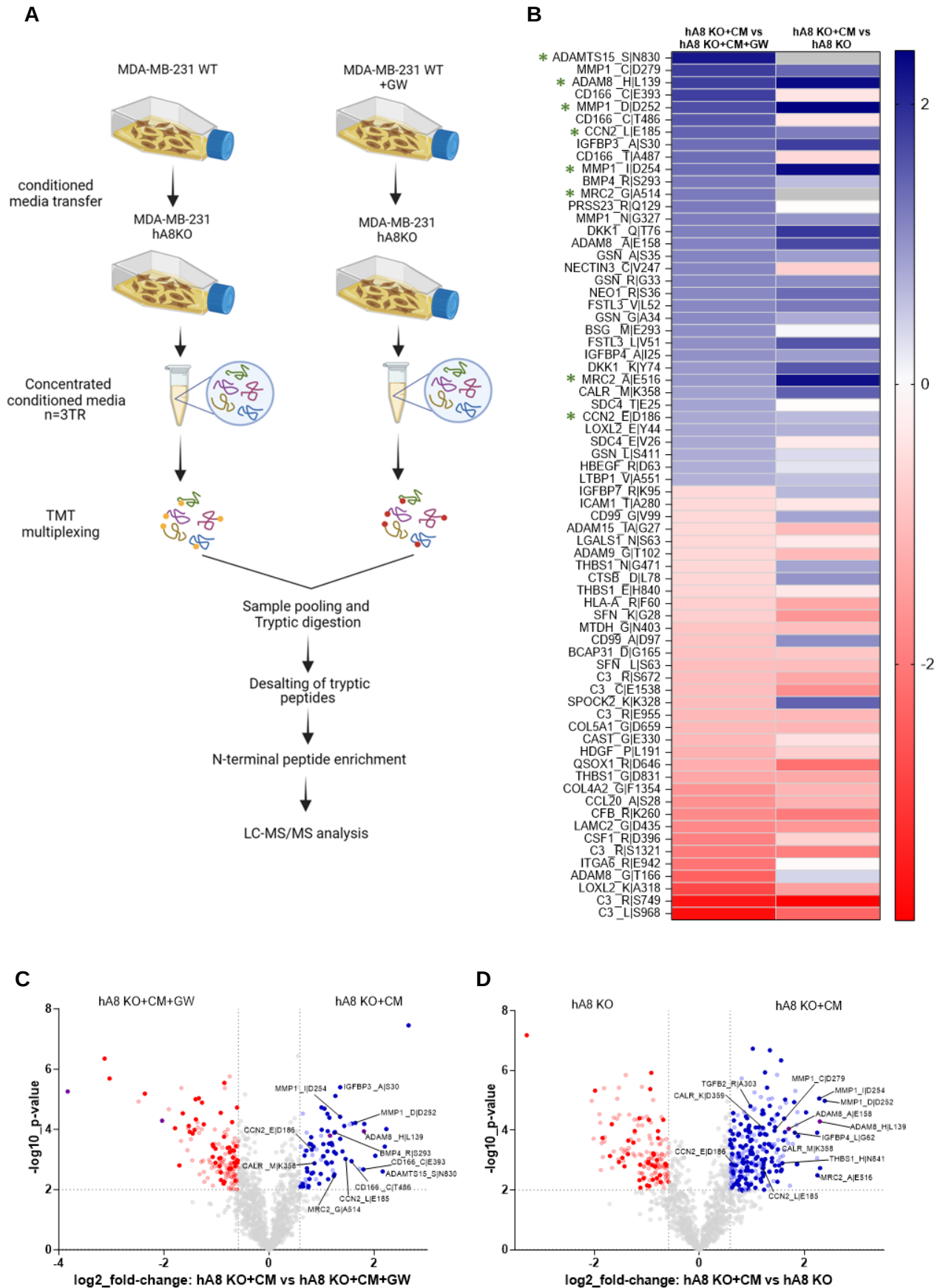


Figure 29: Novel endogenous substrate of ADAM8 and ADAM17

A) For identification of potential new substrates of sADAM17, hA8KO MDA-MB-231 cells were supplied with conditioned media with or without 3μM GW collected from WT cells. The secretome was then harvested and

Results

concentrated. 3 technical replicates from each sample were subjected to TMT-labelling and tryptic digestion. Further, the N-terminally labeled peptides were enriched and subjected to analysis via LC-MS/MS (B) Heatmap generated for the top 35 highest (blue) and least (red) abundant peptides in the hA8 KO cells with CM compared to hA8 KO+CM+GW and hA8 KO. Potential new substrates of sADAM17 are marked with a green asterisk ($\log_2$ fold change ≥ 0.585 or ≤ -0.585) with the p-value ≤ 0.01 . (C) Volcano plot showing all TAILS identified proteolytic events in hA8KO+CM compared to hA8KO+CM+GW and (D) to hA8 KO cells. Gray dotted lines represent threshold values ($\log_2$ fold change $=\pm 0.585$) with the p-value = 0.01. Extracellular peptides are represented with darker blue or red filling.

4. Discussion

The membrane-bound ADAM17 is one of the major metalloproteases to initiate cell signaling via ectodomain shedding. Numerous studies conducted to characterize the membrane-bound ADAM17 concerning its substrates, inhibitors, and adaptor proteins have been reviewed in detail (Calligaris et al., 2021; Scheller et al., 2011; Schumacher & Rose-John, 2022; Zunke & Rose-John, 2017). Regarding maturation, regulation, and substrate selectivity, iRhom1 and iRhom2 play crucial roles in the biological function of membrane-bound ADAM17 (Adrain et al., 2012; Grieve et al., 2017; Maretzky et al., 2013). Therefore, the interactome of membrane-bound ADAM17 is not only restricted to its substrates but also includes the interplay with its adaptor proteins like iRhom1/2. In terms of substrate processing, membrane-bound ADAM17 possesses its individual and shared substrate pool with ADAM10, membrane-type-I matrix metalloprotease (MT1 MMP), and meprin β . The shared pool includes proteins like APP (Asai et al., 2003; Higashi & Miyazaki, 2003; Jefferson et al., 2011), IL6R (Arnold et al., 2017; Riethmueller et al., 2016), etc. Additionally, ADAM8 and ADAM17 have shown an overlapping substrate spectrum including the shedding of TNF-R1 (Bartsch et al., 2010), APP, L-selectin, and TGF- α (Naus et al., 2006).

The complexity of the protease web increases when the proteases themselves get shed from the plasma membrane and released as soluble forms. Interestingly, ADAM10, ADAM17, and MT1-MMP have been shown to shed pro-meprin β resulting in a soluble meprin β that can reach a distant substrate pool (Hahn et al., 2003; Werny et al., 2022; Wichert et al., 2017). In a similar line, other metalloproteases like ADAM9 and 15 have been implicated in the shedding of ADAM10 (Tousseyn et al., 2009). These data insinuated that the reported ADAM17 activities in the blood plasma (Sun et al., 2014) and cerebrospinal fluid (Conrad et al., 2017) could very well be an effect of shed ADAM17. Of note, decreased levels of mature ADAM17 at the cell surface have been reported as a consequence of the exosomal release of ADAM17, which is then able to perform its shedding functions distantly (Groth et al., 2016). However, little was known about the proteolytic release of soluble ADAM17, until the discovery of ADAM8 as a sheddase of membrane-bound ADAM17 (Scharfenberg et al., 2020). The experiments conducted in the TNBC cells (MDA-MB-231), indicated a direct correlation between the expression levels of ADAM8 and ADAM17 (Scharfenberg et al., 2020). Altogether, the overlapping substrate pool and the reported protein-protein interaction hinted towards the interplay between ADAM8 and ADAM17 and their consequent participation in homeostasis and pathophysiology. Based on these observations the main motive of this study was to investigate where the shedding of ADAM17 occurs, further, to identify the involvement of the interacting proteins partners in the regulation of soluble ADAM17, and to explore the functional repertoire of soluble ADAM17.

4.1 Shedding of ADAM17 occurs majorly in the secretory pathway

Typically, ectodomain shedding occurs at the cell surface, however, many metalloproteases have been known to be active in the *trans*-Golgi-network and secretory pathway (Lichtenthaler et al., 2018). In that line, in the preceding research, ADAM8 mediated shedding of ADAM17 for the murine-overexpression constructs was probed in the presence of various ADAM8 specific and cell-permeable inhibitors and the results suggested that shedding of ADAM17 partially occurs in the secretory pathway (Scharfenberg et al., 2020). This suggested that both molecules interact already within the secretory pathway and on the cell surface. However, clear scientific evidence to prove the early interaction of ADAM8 and ADAM17 was still required.

In triple-negative breast cancer, the individual involvement of ADAM17 (McGowan et al., 2013; Zheng et al., 2009) and ADAM8 (Conrad et al., 2018; Cook et al., 2022; Romagnoli et al., 2014) have been investigated in terms of proliferation, invasion, and metastasis. Inhibiting either ADAM8 or ADAM17 leads to anti-metastatic effects. Additionally, decreased levels of sADAM17 in the supernatant of MDA-MD-231 cells without ADAM8 have been observed (Scharfenberg et al., 2020). Therefore, MDA-MB-231 cells endogenously expressing ADAM8 and ADAM17 were employed to further elucidate the colocalization of both the ADAMs via immunofluorescence analysis. Indeed, the immunofluorescence data confirmed the colocalization of ADAM17 and ADAM8 on the plasma membrane and within the secretory pathway. Of note, it was shown that certain stimuli, such as PMA, are required to enhance the cell surface presence of mature ADAM17 (Doedens & Black, 2000; Lorenzen et al., 2016). In the current study, however, sufficient staining was achieved to detect cell surface ADAM17 without external stimulation. For ADAM8, which is usually found on the plasma membrane and the secretory pathway, it was demonstrated that autocatalytic processing is not a prerequisite for its cell surface localization (Schlomann et al., 2002). However, the current immunofluorescence images showed the signals for the catalytically inactive variant of ADAM8 were restricted to the secretory pathway resulting in less colocalization with ADAM17 on the cell surface. However, it cannot be denied that an overall low expression of ADAM8 was observed in hA8 E>Q cells. Together with the data obtained from immunofluorescence images and cell surface biotinylation, the mature ADAM17 was found to be present on the cell surface in all three conditions. However, ADAM8 could not be detected after the biotinylation, perhaps due to antibody's limitation or insufficient biotinylation. Additionally, within the ER, the colocalization of ADAM17 and ADAM8 is independent of the catalytic activity of ADAM8. Perhaps the interaction of ADAM17 and ADAM8 also occurs through their disintegrin domain and the metalloprotease domain of ADAM8 is dispensable for this interaction. Nonetheless, this should be further verified. From the immunofluorescence data, it is evident that ADAM17 and ADAM8 start interacting early in the secretory pathway. The staining within and outside

the ER compartment suggests that ADAM8 and ADAM17 interact throughout the secretory pathway and on the cell surface.

Furthermore, the inhibition of ADAM8-mediated shedding of overexpressed human ADAM17 was only achieved by the membrane permeable metal chelator 1,10-phenanthroline and trafficking inhibitor Brefeldin A (BFA) but not in the presence of EDTA. Since BFA treatment ruptures the ER to Golgi to trafficking mechanism, the autocatalytic maturation of ADAM8 in the *trans*-Golgi-network would not be possible. This could have caused the failure of ADAM8-mediated shedding of ADAM17 in post-ER compartments. This supports the previous study where the shedding of ADAM17 was inhibited only in the secretory pathway (Scharfenberg et al., 2020). Taken together, ADAM8 is indeed one of the major sheddases that soon after its activation, sheds ADAM17 predominantly within the secretory compartments. Additionally, the shedding of ADAM17 in hA8 KO MDA-MB-231 cells resulted in slightly less soluble ADAM17 in the supernatant as compared to the WT cells, which aligns with the previous finding that the absence of ADAM8 does not completely abolish the shedding of ADAM17 (Scharfenberg et al., 2020). The ADAM8 independent shedding however was only significantly inhibited by 1,10-phenanthroline. Perhaps, in the absence of ADAM8, basal shedding of ADAM17 resulting from autocatalytic release of its ectodomain or shedding by other proteases could also occur. However, this needs to be further elucidated by identifying the ADAM8-dependent and independent cleavage sites within the ectodomain of ADAM17 since cleavage fragments created by distinct proteases could be different.

4.2 iRhom1/2 do not participate in the shedding and maturation of sADAM17

The paradigm for the regulation and routine functioning of membrane-bound ADAM17 involves iRhom1 and iRhom2 as regulatory proteins (Adrain & Freeman, 2012; Burzenski et al., 2021; Calligaris et al., 2021). In associated pathologies like inflammation and cancer, mutations in iRhom2 and ADAM17 genes highlighted the importance of their interaction (Düsterhöft et al., 2019). iRhom2 is essential for the forward trafficking and subsequent maturation of ADAM17 in the Golgi network by removal of its pro-domain (Adrain et al., 2012; Christova et al., 2013). Membrane-bound ADAM17 exists in its pro-form and retains in the ER in the absence of iRhom2. On the other hand, in the absence of ADAM17, no iRhom2 is detectable; suggesting that ADAM17 is an important factor in the stabilization of iRhom2 itself (Adrain et al., 2012; McIlwain et al., 2012; Weskamp et al., 2020). Additionally, iRhom1 is reported to be highly upregulated in breast cancer cells, MDA-MB-231 and its deletion or knockdown promotes apoptosis and inhibits migration and invasion *in vitro* (Huang et al., 2018; Zhang et al., 2018). In another study, the silencing of iRhom1 leads to reduced maturation of ADAM17 in MDA-MB-231 cells (Li et al., 2018). In the current study, the shedding of ADAM17 has already been

observed in TNBC cells endogenously expressing iRhom1. Thus, a detailed inspection of iRhom1/2 involvement in the shedding of ADAM17 was further conducted.

Herein, mEF cells endogenously expressing iRhom1/2 were analyzed and compared with its variants without iRhom1/2 and cells stably expressing iRhom2 mutants. To confirm the signal specificity of the mature ADAM17 in the lysate and supernatant, an HA-tag was introduced, C-terminal to the furin cleavage site in the pro-domain of membrane-bound ADAM17. Interestingly, the iRhom1/2^{-/-} mEF cells transfected with mADAM17 PD_HA, a weak band for the matured ADAM17 was observed by employing ADAM17 N-terminal specific antibody (10.3). However, only the pro-form was detected with the antibody against the HA-tag and flag-tag. Intriguingly, in the cell-supernatant of WT and iRhom1/2^{-/-} mEF cells, the mature-soluble form of ADAM17 was detected at ~70kDa. In this study, it was observed that often in the mEF cells system, applying ADAM17 specific antibody for the detection of sADAM17, an unspecific band appears at ~70 kDa, exactly where the signal for sADAM17 appears in the supernatant. This issue was successfully resolved by transfecting the cells with mADAM17 PD_HA, as the detection by antibody against HA-tag confirmed the signal specificity for mature soluble ADAM17 at ~70 kDa. The detection of the soluble ADAM17 with the HA-tag supported the fact that the released ectodomain is processed by furin or other pro-protein convertases in the absence of iRhom1/2. Although iRhom2 is significant for the regulation of ADAM17 in most of the immune cells, the pro-ADAM17 isolated from iRhom2 deficient bone marrow-derived macrophages could be activated by adding recombinant furin (Adrain et al., 2012). This directs towards a possible hypothesis, that in the absence of iRhom1/2, the sheddase of ADAM17, most likely ADAM8, could rescue the ER-retained ADAM17 and promote the trafficking towards the Golgi network. The evident protein-protein interaction and colocalization of ADAM8 and ADAM17 in the early secretory pathway further support this hypothesis. If a pro-form of sADAM17 is released from ER or *cis*-Golgi network, possibly, an iRhom-independent maturation of sADAM17 could occur in the later secretory pathway (Figure 30).

The phosphorylation of the cytoplasmic domain of iRhom2 causes conformational changes in the ADAM17-iRhom2 complex, resulting in the separation of ADAM17 from this complex for the shedding of its substrates (Adrain & Cavadas, 2020; Cavadas et al., 2017; Grieve et al., 2017). Grieve et al., have identified the importance of the N-terminal tail of iRhoms for the stability of ADAM17 at the cell surface to prevent it from lysosomal degradation but for the trafficking and maturation of ADAM17, iRhom homology domain (IRHD) is significant (Grieve et al., 2017). Moreover, the iRhom tail-associated proteins (iTAPs) stabilize this complex further, ADAM17-iRhom2 complex meets its fate via lysosomal degradation (Oikonomidi et al., 2018). Therefore, another possibility could be that the shedding of ADAM17 at the cell surface takes place after its separation from the ADAM17-iRhom2 complex because of the phosphorylation of cytoplasmic N-terminal tail of iRhom2 upon stimulation by GPCR or PMA.

Perhaps apart from keeping ADAM17 in a switched-off state until the stimulation, iRhom2 protects ADAM17 from shedding by forming this stable complex. Possibly, in the current experiments, the endogenous levels of iRhoms in the wild-type cells were not enough to prevent the shedding of overexpressed ADAM17. However, upon stably transfecting the iRhom1/2^{-/-} mEF cells with iRhom2, the shedding of ADAM17 still occurred but with a slightly lower intensity. Thus, iRhoms and their phosphorylation do not play a significant role in the shedding of ADAM17 and the maturation of the soluble ADAM17.

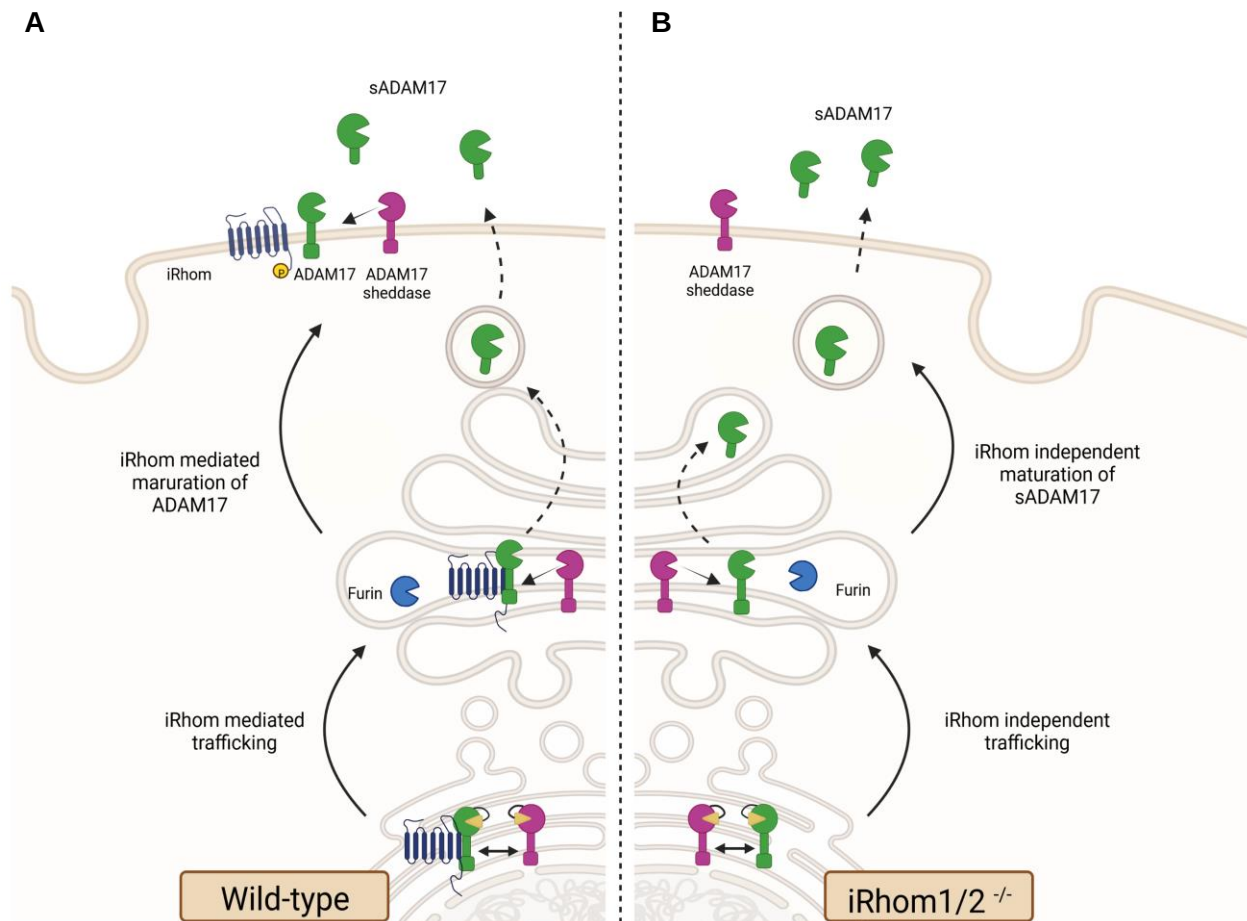


Figure 30: A predicted pathway for iRhom independent shedding of ADAM17

Schematic depiction of possible shedding pathway of (A) Shedding scenario in wildtype cells with iRhom1/2. The interaction of ADAM17 and its sheddase (mostly ADAM8) in the ER. The complex of ADAM17 and ADAM8 gets trafficked to Golgi in an iRhom1/2 mediated way. After the autoactivation of ADAM8 in the TGN, the shedding of ADAM17 occurs predominantly in the secretory pathway. Since the phosphorylation of iRhom2 does not affect shedding at the cell surface, shedding could occur before or after the release of membrane-bound ADAM17 from iRhom/ADAM17 complex. (B) In the absence of iRhom1/2, the proform of ADAM17 gets trafficked forward in the secretory pathway via its strong interaction with ADAM8 or other sheddases. After the autoactivation of ADAM8, the membrane-bound ADAM17 gets shed in the Golgi network. After the shedding, the sADAM17 gets released into the ECM.

4.3 Expression of catalytically active, soluble ADAM17

Several studies showed that the cytoplasmic domain of ADAM17 is dispensable for its stimulated phosphorylation for its activity (Hall & Blobel, 2012; Le Gall et al., 2010). Previously, it was also shown that the complete deletion of the C-terminal domain of ADAM17 results in the loss of shedding activity of ADAM17 without influencing its cellular localization, however, the majority of the cytoplasmic domain is not required for the activity of ADAM17 (Schwarz et al., 2013). The present work showed the release of proteolytically generated mature and soluble ADAM17 in different mammalian cell lines. Furthermore, the proteolytic activity of sADAM17 was confirmed against ADAM17-specific peptides *in vitro*. The persisting proteolytic properties suggested that sADAM17 might have similar cleavage preferences like its membrane-bound counterpart. Additionally, here the shedding of ADAM17 occurs majorly within the secretory pathway. Therefore, sADAM17 can process its substrate in the secretory pathway as well. This was addressed by the generation of overexpressing murine soluble ADAM17 construct. Before analyzing the shedding capacity of the overexpressing sADAM17, the overexpression construct was first characterized in the ADAM10/17^{-/-} HEK293T cells. The secretion of overexpressing sADAM17 was compared to endogenously shed ADAM17 from cells transfected with mADAM17 WT. The sADAM17 in the conditioned media in both approaches showed comparable proteolytic activity against TNF- α based fluorogenic peptide. Additionally, one of the most important post-translational modifications of proteins is glycosylation. Glycosylation can influence the folding, cellular localization, secretion, activation, and stability of proteases (Goettig, 2016). The study performed to check the impact of glycosylation on activation and inhibition of ADAM17, involved the expression of the catalytic domain and ectodomain of ADAM17 in insect and mammalian cells and found out the heavily glycosylated catalytic and ectodomains of ADAM17 to be more efficient in substrate binding (Chavarroche et al., 2014). In the present study, the glycosylation pattern of the overexpressed sADAM17 in the cell-supernatant mimicked that of endogenously shed ADAM17 from the cells transfected with mADAM17 WT. Indeed, soluble ADAM17 carries complex glycans added in the Golgi. Furthermore, the generation of complex glycosylated sADAM17 in the absence of iRhom1 and 2 confirms the independent trafficking of sADAM17 from ER to Golgi while attaining the necessary glycans. Altogether, the characterization data for sADAM17 suggests the release of a properly folded, mature, and proteolytically functional protein into the ECM. This also highlighted that the ectodomain of ADAM17 itself is sufficient for its trafficking through the secretory pathway and its proteolytic activity.

4.4 sADAM17 is capable of shedding EGFR ligands in a substrate-dependent manner

In the previous study performed with recombinant soluble ADAM17, the proteolytic activity was observed against several soluble ECM proteins such as soluble N-cadherin, cystatin-C, and sAPP and shedding function against APP695 (Scharfenberg et al., 2020). However, the shedding of APP695 was suspected to be caused by non-physiological high levels of soluble ADAM17 (Scharfenberg et al., 2020). ADAM17 is actively involved in EGFR signaling, an extremely important signaling pathway for mammalian development and pathologies (Blobel, 2005; Hosur et al., 2018; Sahin et al., 2004). In this study, EGFR ligands that are known substrates of membrane-bound ADAM17 and ADAM10 were investigated in ADAM10/17^{-/-} HEK293T cells. Surprisingly, upon co-expression, soluble ADAM17 displayed persisting shedding capacity against not only AREG and EREG but also against betacellulin which is a known ADAM10 substrate and not cleaved by membrane-bound ADAM17. However, HB-EGF, a well-defined substrate of ADAM17, was not effectively shed by sADAM17 in ADAM10/17^{-/-} HEK293T cells. The conditioned media enriched with proteolytically released sADAM17 revealed AREG, EREG, BTC, and TGF- α as the substrates to sADAM17 also in *trans*. Altogether, this implies that the shedding of EGFR ligands is a time and concentration-dependent process. Correspondingly, sADAM17 persists in shedding capacity in a substrate-dependent manner and possesses a discrete substrate pool but also a shared substrate pool with its membrane-bound counterpart. As mentioned earlier, previous studies reported that most of the cytoplasmic domain of ADAM17 is not required for its rapid activation and its stimulated activity against TNF- α and TGF- α (Hall & Blobel, 2012; Schwarz et al., 2013). However, trimming down the cytoplasmic domain of ADAM17 resulted in a depletion of ADAM17/EGFR signaling in mice (Lora et al., 2021). Though the C-terminal domain is dispensable for the stimulation of ADAM17, its deletion led to drastically decreased levels of ADAM17 and hypomorphic phenotype in mice like heart valve disease and open eye lids (Lora et al., 2021). Nonetheless, the data presented in the current study suggests the constitutive shedding via sADAM17 at similar or even higher levels as compared to membrane-bound ADAM17. Moreover, the ectodomain of ADAM17 could be sufficient for the activation of the signaling pathway independent of its TMD and its cytoplasmic domain.

4.5 sADAM17 sheds AREG and HB-EGF independent of iRhom1/2

iRhoms are not only involved in the regulation of membrane-bound ADAM17, they reportedly control the substrate selectivity of ADAM17 (Maretzky et al., 2013). This selective shedding of the substrates depends on the interaction of iRhom2 and ADAM17 through their trans-membrane domains and juxtamembrane domains (Li et al., 2017; Tang et al., 2020). Of note,

iRhom1 and iRhom2 are reported to regulate ADAM17-mediated EGFR signaling during developmental stages and pathologies in mice (Issuree et al., 2013; Li et al., 2015; Maretzky et al., 2013). Regarding the iRhom2/ADAM17 axis, three studies came out simultaneously, two of which supported the fact that the mutations in the cytoplasmic domain of iRhom2 lead to decreased ADAM17-mediated shedding of AREG (Brooke et al., 2014; Siggs et al., 2014). However, the third study with the cub-mutation in iRhom2 (mutation in the cytoplasmic tail), observed an increased shedding of AREG, but could not identify the source of the elevated shedding (Hosur et al., 2014). According to them, iRhom2 does not regulate EGFR ligands through the regulation of ADAM17, but AREG is a legitimate target of iRhom2 (Burzenski et al., 2021; Hosur et al., 2014). The IRHD domain of iRhom2 is extremely important for ADAM17-mediated EGFR signaling (Adrain & Cavadas, 2020; Düsterhoft et al., 2021; Grieve et al., 2017).

It has been highlighted that in the absence of iRhom2, the shedding of two EGFR ligands; AREG and HB-EGF is disabled but is unaffected for TGF- α (Li et al., 2017; Maretzky et al., 2013). In fact, ADAM17-mediated shedding of TGF- α is dependent on iRhom1 which affects the breast cancer progression (Ji et al., 2022; Li et al., 2018). Due to the significant role of iRhoms in the EGFR signaling via ADAM17, it was investigated whether iRhoms could still participate in the shedding of AREG and HB-EGF via soluble ADAM17. In contrast to previous findings, data in the present study showed in the cells without iRhom1/2, AREG gets significantly shed by sADAM17 only upon co-expression. On the other hand, HB-EGF gets shed when co-expressed with sADAM17 and with proteolytically released sADAM17 is supplied through conditioned media in *trans*. Intriguingly, the shedding of HB-EGF with sADAM17 in the absence of iRhom1/2 contrasted with no shedding at all in the case of ADAM10/17^{-/-} HEK293T cells. From the current study, it can be derived that the shedding of AREG could take place majorly in the secretory pathway. On the contrary, the shedding of HB-EGF occurs in the secretory pathway and more at the cell surface by sADAM17.

As stated earlier, iRhoms and ADAM17 are known to exist in a tight complex on the cell surface, and ADAM17-mediated shedding only takes place upon release of ADAM17 from this complex due to external stimuli like phorbol esters is required for the separation-mediated activation of ADAM17 (Cavadas et al., 2017). Additionally, it has been reported that longer stimulation by PMA results in the downregulation of mature ADAM17 from the cell surface and leads to exosomal release or its internalization and subsequent lysosomal degradation (Groth et al., 2016; Lorenzen et al., 2016). In a more recent proof of concept study, computer-based modeling was employed to deduce the possible mechanism of substrate selectivity of stimulated ADAM17 via transmembrane domains of iRhom1/2. For this, two EGFR ligands, TGF- α and EREG were analyzed for their interaction with iRhom1 or 2 and their consequent interaction with ADAM17 for the shedding (Zhao et al., 2023). However, in the currently shown

data, a basal shedding of HB-EGF without external stimulation via PMA or other physiological stimulators is observed even in the absence of iRhom1/2.

Interestingly, it has been shown that the transmembrane domains (TMD) of ADAM17 and iRhoms are important for their interaction, and by replacing the TMD or the C-terminal domain (CTD) of ADAM17 with that of betacellulin, the maturation and trafficking of ADAM17 becomes independent of iRhoms (Tang et al., 2020). Furthermore, the constitutive shedding activity of ADAM17/BTC chimera, against TGF- α , suggested that iRhoms are not significant for the ectodomain shedding via ADAM17/BTC chimera. It was further proposed that iRhoms are not necessary for the proper folding and functioning of the extracellular domain of ADAM17 (Tang et al., 2020). Taken together, it is obvious that the ectodomain of ADAM17 does not participate in the interaction with iRhom2. The substrate selectivity of sADAM17 is not governed by iRhoms at least in the case of EGFR ligands; AREG and HB-EGF indicating a possible *cis-trans* EGFR-signaling pathway independent of iRhoms. Furthermore, the EGFR signaling in the tissues or cells deficient in iRhoms can be rescued by sADAM17.

4.6 Potential physiological roles of sADAM17 in triple-negative breast cancer

The prognosis of triple-negative breast cancer is difficult since it fails to express the detection markers; Oestrogen receptor (ER), progesterone receptor (PR), and HER2 (Brenton et al., 2005). Hence, targeted therapy in this subtype is extremely challenging. Several studies reported elevated expression of ADAM8 resulting in invasion and metastasis of TNBC (Romagnoli et al., 2014). Whereas, in other studies, ADAM17 was targeted with inhibitor antibodies to inhibit EGFR signaling and suppress tumor growth (Caiazza et al., 2015; Saha et al., 2022). In a recent study performed with co-cultures, it was reported that a paracrine EGFR signaling mediated feedback loop induces cell invasion of aggressive breast cancer cells. Where the ADAM17 expressed by cancer cells mediates EGFR signaling by the release of HB-EGF or AREG which in turn induces the secretion of chemokines by macrophages that promotes cell invasion (Gnosa et al., 2022). This observation of paracrine signaling between the two different cell populations can be substantiated by the current observation of EGFR ligand shedding via sADAM17 in *cis* and *trans*. The reported cell-invasion properties could very well be facilitated by sADAM17 present in the culture medium.

In an attempt to identify the potential serum markers, the secretome from the 3D culture of MDA-MB-231 cells was also analyzed for the abundance of secreted proteins (Ziegler et al., 2016). This mass-spectroscopy analysis identified proteins belonging to different groups like ECM, growth factors, proteases, and their inhibitor and IGFBPs (Ziegler et al., 2016). However, a thorough analysis of the cleavage events that led to various cellular processes like angiogenesis, invasion, metastasis, etc. was yet to be done.

In the present work, the secretome of variants of MDA-MB-231 cells was analyzed via the N-terminomics TAILS approach. Upon comparing WT cells to hA8 KO and hA8 E>Q cells, several peptides were identified to be potentially generated by ADAM8-mediated activity. ADAM8 can autocatalytically cleave off its pro-domain for its activation, this active form can be further processed and release the metalloprotease domain into the extracellular matrix, leaving a remnant form attached to the membrane (Schlomann et al., 2002). The soluble ADAM8 comprising the metalloprotease domain is known to further interact and cleave the ECM proteins in the tumour microenvironment (Conrad et al., 2019; Conrad et al., 2018). From the currently obtained data, in HLA-A or MHC-I, the cleavage was recognized within the alpha 1 chain. HLA-A presents the antigen on the cell surface and the cleavage within the alpha 1 chain could disrupt the peptide binding pocket of HLA-A. Of note, the alpha 1 chain of HLA-A is required to interact with a component of the peptide loading complex (PLC), CALR to achieve proper folding and to create an immune response (Dersh et al., 2021; Rizvi et al., 2011). The C-terminal domain of CALR is responsible for mediating the Ca²⁺-dependent pathway (Nakamura et al., 2001) and phagocytosis of apoptotic cells (Wijeyesakere et al., 2016). Additionally, the frameshift mutations in the C-terminal domain of CALR result in the loss of ER retention signal KDEL, which ultimately affects the peptide loading of HLA-A (Arshad & Cresswell, 2018). Interestingly, from current data, two cleavages of 17 amino acids before the frame shift mutation site were identified within the C-terminal domain of CALR. For THBS1, bone morphogenic protein 1 (BMP1) cleaves the protein into two fragments that affect the activation of TGFβ via THBS1 (Anastasi et al., 2020). Here, N-terminomics identified a cleavage within the calcium-binding domain which might affect cell adhesion and motility. A C-terminal cleavage was also seen in IGFBP3. On the other hand, in LTBP1, the cleavage site was found to be more N-terminal between EGF-like 2 and TB1 domain, far from the TGF-β binding site (Gleizes et al., 1996; Saharinen et al., 1996). Intriguingly, several MMP1 peptides were found to be upregulated in the secretome of hA8 E>Q cells as compared to hA8 KO cells. Perhaps, ADAM8 possesses additional non-proteolytic functions and contributes within the ECM via upregulation of certain proteins, hence the activation of ADAM8 is not necessary. Moreover, some cleavage events like cleavage within the propeptide of ADAM8 (H|L139) and cleavage in the spacer region of ADAMTS15 (S|N830) appeared to be upregulated in the WT cells against the cells with GW as ADAM17 inhibitor.

Furthermore, for the identification of potential sADAM17 substrates, the hA8 KO cells were treated with conditioned media (CM) enriched with proteolytically released sADAM17 by WT cells. The secretomes of cells treated with CM were compared to the cells without CM and cells with CM and GW. Here, the upregulated cleaved peptide in the secretome of CM treated cells was supposed to have major cleavage events from sADAM17 and a few events by ADAM8 and ADAM17. With this approach, a few more interesting extracellular proteins were

identified as potential sADAM17 substrates like CCN2, ADAMTS15, MRC2, and ADAM8. CCN2 also known as connective tissue growth factor (CTGF) is highly expressed in TNBC cells and is reported to contribute to augmenting migration of TNBC cells via the integrin $\alpha\text{v}\beta\text{3}$ -ERK1/2 pathway (Chen et al., 2007). Moreover, upon binding to integrin $\alpha\text{v}\beta\text{3}$, CCN2 induces upregulation of Glut3 resulting in poor survival rates (Kim et al., 2021). Of note, full-length CCN2 is a soluble protein consisting of four domains and needs to be proteolytically processed to release an active fragment. This processing takes place by cleavage mediated by MMPs (7,8,12 and 13) in the so-called “Hinge region” (Hashimoto et al., 2002; Kaasboll et al., 2018). Besides known cleavage sites, the present data identified two new additional cleavage events within the hinge region, adjacent to the MMP13 cleavage site. Furthermore, ADAMTS15 is a secreted ECM protein that depending upon the tumour microenvironment can function pro or anti-metastatic way (Kelwick et al., 2015). From N-terminomics data a cleavage within the spacer region was noted, however, how this cleavage can affect the ADAMTS15 activity needs to be elucidated. For MRC2, also known as Endo180, two cleavages were found closer to the N-terminal between C-lectin 2 and 3 domains. Of note, the soluble form of MRC2 is reported to be an efficient marker in the metastatic TNBC cells (Palmieri et al., 2013). Recently, it was reported that membrane-bound ADAM17 sheds MRC2 in an iRhom1-dependent manner (Tushaus et al., 2021). Apart from the above-mentioned cleavage events, several sites in CD166 (ALCAM) were also recognized. CD166 is already a known substrate of membrane-bound ADAM17 (Bech-Serra et al., 2006; Santiago-Josefat et al., 2007). The functional and reactome enrichment analysis of the upregulated extracellular proteins revealed the involvement of most of the proteins like THBS1, PXDN, IGFBP3, CALR, LTBP1, and ADAM8 in ECM organization. Several other functions like binding to fibronectin, integrin binding, and Ca^{2+} binding also suggest the role of these proteins in the migration and mobility of the cells.

4.7 Summary and Outlook

In sum, considering the results obtained from *in vitro* experiments, the shedding of ADAM17 is not just a process to downregulate cell surface ADAM17, rather it enables soluble ADAM17 to access the substrates in close cellular vicinity and at a distance as well. ADAM8 is the major sheddase of ADAM17, however with the observation of ADAM8 independent shedding of ADAM17, further investigation to identify the cleavage site within the ectodomain would clarify the processing of ADAM17 by different sheddases. Nonetheless, in therapeutic terms, the activity of sADAM17 in pathologies can be reduced by targeting ADAM8 activity. The iRhom independent trafficking and maturation of soluble ADAM17 hints towards a different possible mechanism for the regulation of sADAM17 which needs to be further elucidated. Regarding the substrate repertoire, the *in vitro* experiments exhibited that sADAM17 certainly does not require iRhoms for substrate selectivity and processing. However, the mechanism of substrate accessibility could be entirely different than that of membrane-bound ADAM17. Additionally, the sADAM17-mediated shedding occurs in a substrate-dependent manner within the secretory pathway and on the cell surface in *cis* and *trans*. N-terminomics TAILS approach significantly facilitated in identification of putative novel substrates of sADAM17 in aggressive breast cancer cells. This is another step towards a better understanding of the potential roles of sADAM17 in breast cancer-associated malignancies. However, the identified cleavage events need to be further verified via *in vitro* assays and LC/MS analysis. Further *in vitro* tests with the ADAM8 KO and ADAM17 KO cells for the analysis of migration and invasion capacity in the presence or absence of sADAM17 must be performed via scratch test or co-culture respectively. In addition to that, further *in vivo* or *ex vivo* experiments with appropriate mouse models would provide a bigger picture of the physiological relevance of sADAM17 as demonstrated for its membrane-bound counterpart. Figure 31 illustrates the possible role of sADAM17 in the malignancies associated with breast cancer via signaling in *cis* (Figure 31 A), signaling in *trans* by shedding the EGFR ligands in *cis* and *trans* (Figure 31B), and potential role in the ECM degradation and remodeling (Figure 31C).

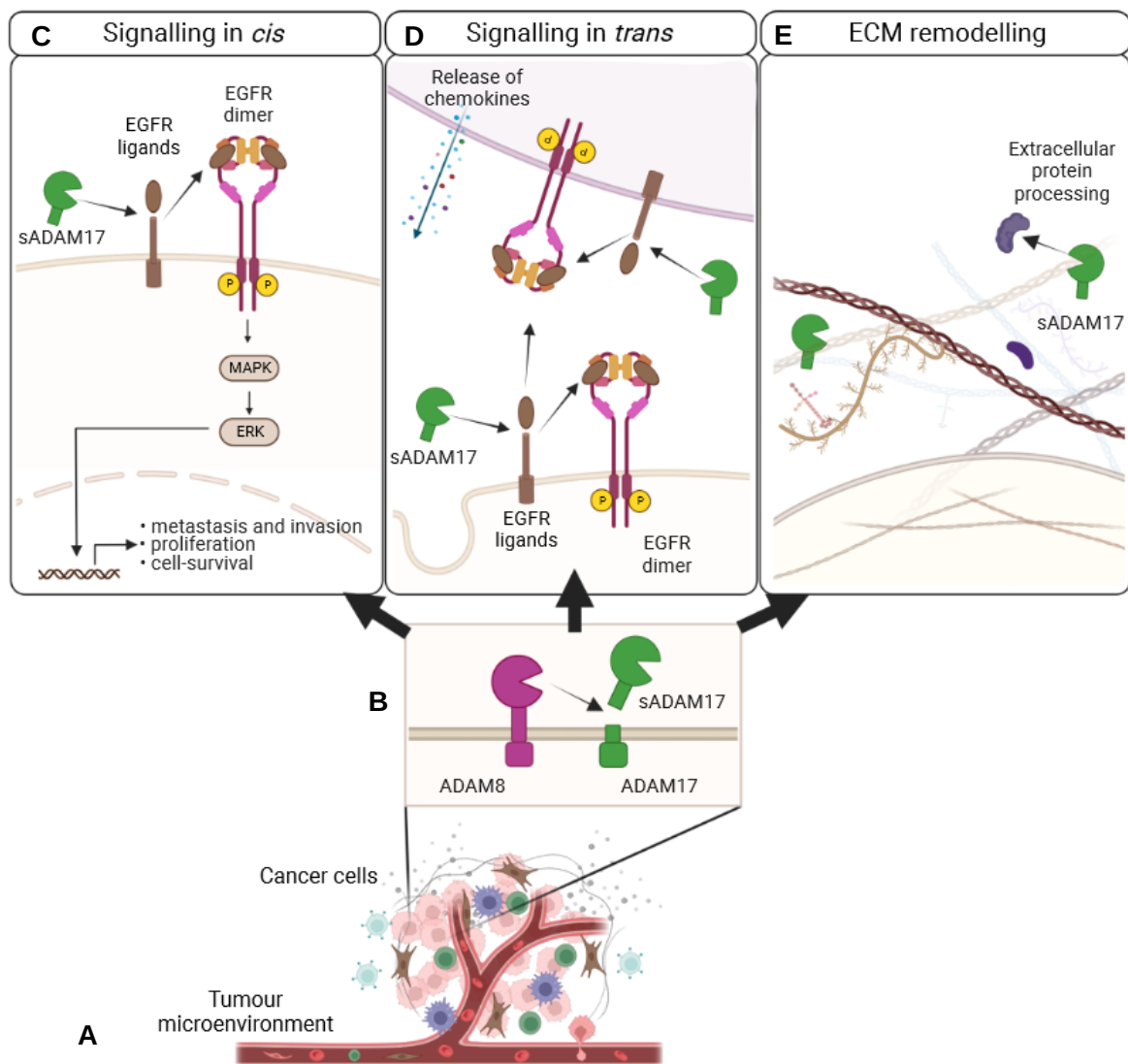


Figure 31: Potential Physiological relevance of sADAM17

Schematic illustration of (A) Tumor microenvironment consisting of cancer cells, immune cells, and blood vessels with invading cancer cells (B) ADAM8 mediated shedding of ADAM17 in the cancer cells (C) sADAM17-mediated EGFR signaling in *cis*, initiating metastasis, proliferation, and invasion (D) sADAM17-mediated signaling in *trans* via ligand shedding on the same cell and distant cell (E) sADAM17-mediated ECM remodeling via cleavage of soluble ECM proteins.

5. References

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6. Supplementary Information

Supplementary table 1: GO functions, Reactome, Kegg Pathway and Biological process analysis via Metascpae for top 30 proteins upregulated in WT MDA-MB-231 cells against hA8 KO, hA8 E>Q cells

Term	Description	LogP	Symbols
R-HSA-1474244	Extracellular matrix organization	-12.71	ADAM8, BMP4, CTSB,CTSL,ITGB4, LTBP1,THBS1,TIMP2,PXDN,LTBP4
M3468	NABA ECM REGULATORS	-5.46	ADAM8, CTSB, CTSL,TGM2,TIMP2
R-HSA-1474228	Degradation of the extracellular matrix	-4.96	ADAM8, CTSB,CTSL,TIMP2
M5885	NABA MATRISOME ASSOCIATED	-4.09	ADAM8, BMP4,CTSB,CTSL,TGM2, TIMP2
R-HSA-6798695	Neutrophil degranulation	-3.99	ADAM8, CTSB,GRN,QSOX1,TIMP2
M3008	NABA ECMGLYCOPROTEINS	-10.86	CCN2,IGFBP3,IGFBP7,LTBP1,THBS 1, PXDN,LTBP4,SLIT2
M5884	NABA CORE MATRISOME	-9.69	CCN2,IGFBP3,IGFBP7,LTBP1,THBS 1, PXDN,LTBP4,SLIT2
GO:0001558	regulation of cell growth	-5.53	APOE,IGFBP3,IGFBP7,PPT1,LTBP4, SLIT2
GO:0040008	regulation of growth	-4.57	APOE,IGFBP3,IGFBP7,PPT1,LTBP4, SLIT2
R-HSA-8957275	Post-translational protein phosphorylation	-9.01	APOE,BMP4,IGFBP3,IGFBP7,LTBP1, QSOX1
R-HSA-381426	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	-8.62	APOE,BMP4,IGFBP3,IGFBP7,LTBP1, QSOX1
GO:0048660	regulation of smooth muscle cell proliferation	-4.62	APOE,BMP4,IGFBP3,THBS1
GO:0048662	negative regulation of smooth muscle cell proliferation	-4.34	APOE,BMP4,IGFBP3
GO:0050680	negative regulation of epithelial cell proliferation	-3.18	APOE,BMP4,THBS1
GO:0050678	regulation of epithelial cell proliferation	-3.18	APOE,BMP4,GRN,THBS1
GO:0001936	regulation of endothelial cell proliferation	-3.12	APOE,BMP4,THBS1
GO:0070374	positive regulation of ERK1 and ERK2 cascade	-2.89	APOE,BMP4,CCN2
GO:0050866	negative regulation of cell activation	-2.86	APOE,BMP4,GRN
GO:0048871	multicellular organismal-level homeostasis	-2.51	APOE,BMP4,CCN2,NEO1
GO:0070372	regulation of ERK1 and ERK2 cascade	-2.44	APOE,BMP4,CCN2
GO:0043065	positive regulation of apoptotic process	-8.76	ADAM8,BMP4,CTSL,GRN,IGFBP3, TGM2,THBS1,SLIT2,BCAP31
GO:0043068	positive regulation of programmed cell death	-8.60	ADAM8,BMP4,CTSL,GRN,IGFBP3, TGM2,THBS1,SLIT2,BCAP31

GO:0030162	regulation of proteolysis	-6.95	ADAM8,APOE,CTSL,GRN,LRP1,THBS1,TIMP2,BCAP31
GO:0052547	regulation of peptidase activity	-6.26	CTSL,GRN,LRP1,THBS1,TIMP2,BCAP31
R-HSA-983170	Antigen Presentation: Folding, assembly and peptide loading of class I MHC	-5.50	CALR,HLA-A,BCAP31
GO:0051336	regulation of hydrolase activity	-5.07	CTSL,GRN,LRP1,TGM2,THBS1,TIMP2,BCAP31
GO:0045862	positive regulation of proteolysis	-4.65	ADAM8,APOE,CTSL,GRN,BCAP31
hsa05165	Human papillomavirus infection	-3.51	HLA-A,ITGB4,THBS1,BCAP31
GO:2001235	positive regulation of apoptotic signaling pathway	-3.40	CTSL,THBS1,BCAP31
GO:2001233	regulation of apoptotic signaling pathway	-3.21	BMP4,CTSL,THBS1,BCAP31
GO:0010952	positive regulation of peptidase activity	-3.20	CTSL,GRN,BCAP31
GO:0051345	positive regulation of hydrolase activity	-2.84	CTSL,GRN,TGM2,BCAP31
GO:0034976	response to endoplasmic reticulum stress	-2.83	CALR,THBS1,BCAP31
GO:0052548	regulation of endopeptidase activity	-2.54	THBS1,TIMP2,BCAP31
GO:0048514	blood vessel morphogenesis	-8.17	ADAM8,APOE,BMP4,CCN2,LRP1,THBS1,PXDN,SLIT2
GO:0035239	tube morphogenesis	-7.90	ADAM8,APOE,BMP4,CCN2,LRP1,TGM2,THBS1,PXDN,SLIT2
GO:0001568	blood vessel development	-7.50	ADAM8,APOE,BMP4,CCN2,LRP1,THBS1,PXDN,SLIT2
GO:0001944	vasculature development	-7.38	ADAM8,APOE,BMP4,CCN2,LRP1,THBS1,PXDN,SLIT2
GO:0014912	negative regulation of smooth muscle cell migration	-7.25	BMP4,IGFBP3,LRP1,SLIT2
GO:0030336	negative regulation of cell migration	-7.19	APOE,BMP4,CALR,IGFBP3,LRP1,THBS1,SLIT2
GO:2000146	negative regulation of cell motility	-7.07	APOE,BMP4,CALR,IGFBP3,LRP1,THBS1,SLIT2
GO:0040013	negative regulation of locomotion	-6.82	APOE,BMP4,CALR,IGFBP3,LRP1,THBS1,SLIT2
GO:0001525	angiogenesis	-6.07	ADAM8,BMP4,CCN2,THBS1,PXDN,SLIT2
GO:0014910	regulation of smooth muscle cell migration	-5.75	BMP4,IGFBP3,LRP1,SLIT2
GO:0002040	sprouting angiogenesis	-4.54	BMP4,THBS1,SLIT2
GO:0003170	heart valve development	-4.28	BMP4,SLIT2,DKK1
GO:0002683	negative regulation of immune system process	-3.84	BMP4,GRN,HLA-A,THBS1,SLIT2
GO:0090288	negative regulation of cellular response to growth factor stimulus	-3.57	THBS1,SLIT2,DKK1
GO:0061138	morphogenesis of a branching epithelium	-3.25	BMP4,TGM2,SLIT2

GO:0001763	morphogenesis of a branching structure	-3.17	BMP4,TGM2,SLIT2
GO:0010720	positive regulation of cell development	-3.04	ADAM8, BMP4, TGM2, SLIT2
GO:0007507	heart development	-2.64	BMP4, CALR,SLIT2,DKK1
GO:0032956	regulation of actin cytoskeleton organization	-2.35	CCN2, LRP1,SLIT2
GO:0006935	chemotaxis	-2.34	ADAM8,BMP4,SLIT2
GO:0042330	taxis	-2.33	ADAM8,BMP4,SLIT2
GO:0007420	brain development	-2.28	BMP4,PPT1,SLIT2,DKK1
GO:0040011	locomotion	-2.27	ADAM8,BMP4,SLIT2
GO:0032970	regulation of actin filament-based process	-2.21	CCN2, LRP1, SLIT2
GO:0060322	head development	-2.18	BMP4, PPT1,SLIT2,DKK1
GO:0030900	forebrain development	-2.13	BMP4,SLIT2,DKK1
GO:0002009	morphogenesis of an epithelium	-2.00	BMP4, TGM2,SLIT2
GO:0090092	regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	-7.87	BMP4, LRP1,LTBP1,NEO1,THBS1, LTBP4,DKK1
GO:0090287	regulation of cellular response to growth factor stimulus	-7.32	BMP4, LTBP1,NEO1,THBS1,LTBP4, SLIT2,DKK1
hsa04350	TGF-beta signaling pathway	-5.41	BMP4, LTBP1,NEO1,THBS1
R-HSA-2129379	Molecules associated with elastic fibres	-5.18	BMP4, LTBP1,LTBP4
GO:0071634	regulation of transforming growth factor beta production	-5.01	ADAM8,LTBP1,THBS1
R-HSA-1566948	Elastic fibre formation	-4.95	BMP4,LTBP1,LTBP4
WP560	TGF-beta receptor signaling	-4.65	BMP4,LTBP1,THBS1
WP4816	TGF-beta receptor signaling in skeletal dysplasias	-4.56	BMP4,LTBP1,THBS1
GO:0090100	Positive regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	-3.83	BMP4,NEO1,THBS1
GO:0007167	enzyme-linked receptor protein signaling pathway	-3.50	BMP4,CCN2,LRP1,LTBP1,LTBP4
GO:0017015	regulation of transforming growth factor beta receptor signaling pathway	-3.28	LTBP1,THBS1,LTBP4
GO:1903844	regulation of cellular response to transforming growth factor beta stimulus	-3.25	LTBP1,THBS1,LTBP4
GO:0007178	transmembrane receptor protein serine/threonine kinase signaling pathway	-3.02	BMP4,LTBP1,LTBP4
GO:0071363	cellular response to growth factor stimulus	-2.89	BMP4,CCN2,THBS1,LTBP4
GO:0070848	response to growth factor	-2.79	BMP4,CCN2,THBS1,LTBP4
GO:0030003	intracellular monoatomic cation homeostasis	-6.84	APOE,CALR,GRN,NEO1,PPT1, TGM2,BCAP31
GO:0006873	intracellular monoatomic ion homeostasis	-6.78	APOE,CALR,GRN,NEO1,PPT1, TGM2,BCAP31

GO:0055080	monoatomic cation homeostasis	-6.38	APOE,CALR,GRN,NEO1,PPT1,TGM2,BCAP31
GO:0050801	monoatomic ion homeostasis	-6.32	APOE,CALR,GRN,NEO1,PPT1,TGM2,BCAP31
GO:0055082	intracellular chemical homeostasis	-5.98	APOE,CALR,GRN,NEO1,PPT1,TGM2,BCAP31
GO:0019725	cellular homeostasis	-5.48	APOE,CALR,GRN,NEO1,PPT1,TGM2,BCAP31
GO:0006874	intracellular calcium ion homeostasis	-4.36	APOE,CALR,TGM2,BCAP31
GO:0098771	inorganic ion homeostasis	-4.23	APOE,CALR,NEO1,TGM2,BCAP31
GO:0055074	calcium ion homeostasis	-4.15	APOE,CALR,TGM2,BCAP31
GO:0051235	maintenance of location	-3.18	APOE,CALR,LTBP1
GO:0043410	positive regulation of MAPK cascade	-6.49	ADAM8,APOE,BMP4,CCN2,IGFBP3,THBS1,DKK1
GO:0043408	regulation of MAPK cascade	-5.47	ADAM8,APOE,BMP4,CCN2,IGFBP3,THBS1,DKK1
GO:0043405	regulation of MAP kinase activity	-4.94	ADAM8,APOE,THBS1,DKK1
GO:0050865	regulation of cell activation	-4.43	ADAM8,APOE,BMP4,GRN,HLA-A,THBS1
GO:0043406	positive regulation of MAP kinase activity	-4.04	ADAM8,THBS1,DKK1
GO:0071900	regulation of protein serine/threonine kinase activity	-3.76	ADAM8,APOE,THBS1,DKK1
GO:0002694	regulation of leukocyte activation	-3.55	ADAM8,BMP4,GRN,HLA-A,THBS1
GO:0071902	positive regulation of protein serine/threonine kinase activity	-3.45	ADAM8,THBS1,DKK1
GO:0051347	positive regulation of transferase activity	-3.15	ADAM8,APOE,THBS1,DKK1
GO:0045785	positive regulation of cell adhesion	-2.90	ADAM8,CALR,HLA-A,TGM2
GO:0001819	positive regulation of cytokine production	-2.85	ADAM8,HLA-A,NMB,THBS1
GO:0045859	regulation of protein kinase activity	-2.84	ADAM8,APOE,THBS1,DKK1
GO:0070663	regulation of leukocyte proliferation	-2.64	BMP4,HLA-A,NMB
GO:0043549	regulation of kinase activity	-2.61	ADAM8,APOE,THBS1,DKK1
GO:0045860	positive regulation of protein kinase activity	-2.57	ADAM8,THBS1,DKK1
GO:0001934	positive regulation of protein phosphorylation	-2.54	ADAM8,BMP4,THBS1,DKK1
GO:0042327	positive regulation of phosphorylation	-2.41	ADAM8,BMP4,THBS1,DKK1
GO:0033674	positive regulation of kinase activity	-2.36	ADAM8,THBS1,DKK1
GO:0051338	regulation of transferase activity	-2.34	ADAM8,APOE,THBS1,DKK1
GO:0010562	positive regulation of phosphorus metabolic process	-2.26	ADAM8,BMP4,THBS1,DKK1

GO:0045937	positive regulation of phosphate metabolic process	-2.26	ADAM8,BMP4,THBS1,DKK1
GO:0002696	positive regulation of leukocyte activation	-2.21	ADAM8,HLA-A,THBS1
GO:0050863	regulation of T cell activation	-2.21	ADAM8,BMP4,HLA-A
GO:1903037	regulation of leukocyte cell-cell adhesion	-2.20	ADAM8,BMP4,HLA-A
GO:0050867	positive regulation of cell activation	-2.16	ADAM8,HLA-A,THBS1
GO:0030100	regulation of endocytosis	-6.44	APOE,CALR,LRP1,PPT1,TGM2,DKK1
GO:0006897	endocytosis	-6.25	APOE,CTSL,LRP1,PPT1,TGM2,THBS1,MRC2
GO:0098657	import into cell	-5.34	APOE,CTSL,LRP1,PPT1,TGM2,THBS1,MRC2
R-HSA-2173782	Binding and Uptake of Ligands by Scavenger Receptors	-5.01	APOE,CALR,LRP1
GO:0043393	regulation of protein binding	-4.94	APOE,BMP4,LRP1,DKK1
GO:0060627	regulation of vesicle-mediated transport	-4.94	APOE,CALR,LRP1,PPT1,TGM2,DKK1
GO:0032368	regulation of lipid transport	-4.81	APOE,LRP1,NMB,THBS1
GO:0045807	positive regulation of endocytosis	-4.79	APOE,CALR,LRP1,PPT1
GO:0006898	receptor-mediated endocytosis	-4.70	APOE,CTSL,LRP1,PPT1
GO:0032092	positive regulation of protein binding	-4.52	APOE,BMP4,LRP1
GO:1905952	regulation of lipid localization	-4.51	APOE,LRP1,NMB,THBS1
GO:0048844	artery morphogenesis	-4.34	APOE,BMP4,LRP1
GO:0032370	positive regulation of lipid transport	-4.07	APOE,LRP1,NMB
GO:0051098	regulation of binding	-3.90	APOE,BMP4,LRP1,DKK1
GO:0060840	artery development	-3.86	APOE,BMP4,LRP1
GO:1905954	positive regulation of lipid localization	-3.74	APOE,LRP1,NMB
GO:0051099	positive regulation of binding	-3.57	APOE,BMP4,LRP1
GO:0030178	negative regulation of Wnt signaling pathway	-3.18	APOE,LRP1,DKK1
GO:0090101	negative regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	-3.14	LRP1,LTBP1,DKK1
WP5124	Alzheimer's disease	-2.66	APOE,LRP1,DKK1
WP2059	Alzheimer's disease and miRNA effects	-2.39	APOE,LRP1,DKK1
GO:0030111	regulation of Wnt signaling pathway	-2.35	APOE,LRP1,DKK1
WP5323	Fatty Acids and Lipoproteins Transport in Hepatocytes	-2.21	APOE,LRP1,MRC2
hsa05010	Alzheimer disease	-2.20	APOE,LRP1,DKK1
GO:0061024	membrane organization	-2.19	APOE,CTSL,PPT1,THBS1
hsa04145	Phagosome	-6.42	CALR,CTSL,HLA-A,THBS1,MRC2

R-HSA-2022090	Assembly of collagen fibrils and other multimeric structures	-6.41	CTSB,CTSL,ITGB4,PXDN
hsa04612	Antigen processing and presentation	-5.98	CALR,CTSB,CTSL,HLA-A
R-HSA-1474290	Collagen formation	-5.73	CTSB,CTSL,ITGB4,PXDN
R-HSA-1236975	Antigen processing-Cross presentation	-5.46	CALR,CTSL,HLA-A,MRC2
GO:0030574	collagen catabolic process	-5.04	CTSB,CTSL,MRC2
GO:0032963	collagen metabolic process	-4.48	CTSB,CTSL,MRC2
R-HSA-983169	Class I MHC mediated antigen processing & presentation	-4.47	CALR,CTSL,HLA-A,MRC2,BCAP31
GO:0048002	antigen processing and presentation of peptide antigen	-4.36	CALR,CTSL,HLA-A
R-HSA-1280218	Adaptive Immune System	-4.03	CALR,CTSB,CTSL,HLA-A,MRC2,BCAP31
GO:0019882	antigen processing and presentation	-3.84	CALR,CTSL,HLA-A
WP4217	Ebola virus infection in host	-3.55	CTSB,CTSL,HLA-A
hsa04142	Lysosome	-3.52	CTSB,CTSL,PPT1
GO:0030163	protein catabolic process	-3.22	APOE,CALR,CTSB,CTSL,PPT1
GO:0019058	viral life cycle	-3.14	APOE,CTSB,CTSL
WP5115	Network map of SARS-CoV-2 signaling pathway	-2.89	CTSB,CTSL,IGFBP3
GO:0010817	regulation of hormone levels	-2.73	CTSB,CTSL,NMB,LTBP4
GO:0016032	viral process	-2.73	APOE,CTSB,CTSL
GO:0044403	biological process involved in symbiotic interaction	-2.63	APOE,CTSB,CTSL
GO:0051043	regulation of membrane protein ectodomain proteolysis	-5.76	ADAM8,APOE,TIMP2
GO:0043523	regulation of neuron apoptotic process	-5.51	ADAM8,APOE,GRN,PPT1,DKK1
GO:0043524	negative regulation of neuron apoptotic process	-4.70	ADAM8,APOE,GRN,PPT1
GO:0042176	regulation of protein catabolic process	-4.57	ADAM8,APOE,LRP1,TIMP2,BCAP31
GO:0045732	positive regulation of protein catabolic process	-4.27	ADAM8,APOE,LRP1,BCAP31
GO:0051223	regulation of protein transport	-3.88	ADAM8,APOE,BMP4,NEO1,BCAP31
GO:0070201	regulation of establishment of protein localization	-3.78	ADAM8,APOE,BMP4,NEO1,BCAP31
GO:1903531	negative regulation of secretion by cell	-3.34	APOE,NEO1,NMB
GO:0051048	negative regulation of secretion	-3.19	APOE,NEO1,NMB
GO:0045088	regulation of innate immune response	-3.11	ADAM8,APOE,GRN,HLA-A
GO:0051051	negative regulation of transport	-2.95	APOE,NEO1,NMB,THBS1
GO:1903829	positive regulation of protein localization	-2.92	ADAM8,BMP4,LRP1,BCAP31
GO:0002831	regulation of response to biotic stimulus	-2.81	ADAM8,APOE,GRN,HLA-A

GO:1903530	regulation of secretion by cell	-2.65	ADAM8,APOE,NEO1,NMB
GO:0050708	regulation of protein secretion	-2.64	ADAM8,APOE,NEO1
GO:0009896	positive regulation of catabolic process	-2.58	ADAM8,APOE,LRP1,BCAP31
GO:0051046	regulation of secretion	-2.51	ADAM8,APOE,NEO1,NMB
GO:0031348	negative regulation of defense response	-2.41	APOE,GRN,HLA-A
GO:0031329	regulation of cellular catabolic process	-2.27	ADAM8,APOE,QSOX1,TIMP2
GO:0050727	regulation of inflammatory response	-2.08	ADAM8,APOE,GRN
GO:0010594	regulation of endothelial cell migration	-5.52	APOE,CALR,GRN,THBS1,SLIT2
GO:0032102	negative regulation of response to external stimulus	-5.19	APOE,GRN,HLA-A,NEO1,THBS1,SLIT2
GO:0010632	regulation of epithelial cell migration	-5.01	APOE,CALR,GRN,THBS1,SLIT2
GO:0010977	negative regulation of neuron projection development	-4.96	APOE,NEO1,SLIT2,DKK1
GO:1903034	regulation of response to wounding	-4.60	APOE,GRN,NEO1,THBS1
GO:0031345	negative regulation of cell projection organization	-4.42	APOE,NEO1,SLIT2,DKK1
GO:0051129	negative regulation of cellular component organization	-4.21	APOE,BMP4,NEO1,THBS1,SLIT2,DKK1
GO:0010975	regulation of neuron projection development	-4.12	APOE,GRN,NEO1,SLIT2,DKK1
GO:0010596	negative regulation of endothelial cell migration	-3.89	APOE,THBS1,SLIT2
GO:0010633	negative regulation of epithelial cell migration	-3.69	APOE,THBS1,SLIT2
GO:0001933	negative regulation of protein phosphorylation	-3.65	APOE,IGFBP3,SLIT2,DKK1
GO:0042326	negative regulation of phosphorylation	-3.53	APOE,IGFBP3,SLIT2,DKK1
GO:0120035	regulation of plasma membrane-bounded cell projection organization	-3.38	APOE,GRN,NEO1,SLIT2,DKK1
GO:0031344	regulation of cell projection organization	-3.33	APOE,GRN,NEO1,SLIT2,DKK1
GO:0045936	negative regulation of phosphate metabolic process	-3.31	APOE,IGFBP3,SLIT2,DKK1
GO:0010563	negative regulation of phosphorus metabolic process	-3.30	APOE,IGFBP3,SLIT2,DKK1
GO:0031400	negative regulation of protein modification process	-3.06	APOE,IGFBP3,SLIT2,DKK1
GO:0050803	regulation of synapse structure or activity	-2.72	APOE,PPT1,DKK1
GO:0003018	vascular process in circulatory system	-2.63	APOE,LRP1,SLIT2
GO:0007611	learning or memory	-2.61	APOE,PPT1,DKK1
GO:1902532	negative regulation of intracellular signal transduction	-2.59	APOE,LRP1,THBS1,SLIT2
GO:0007610	behavior	-2.53	APOE,PPT1,THBS1,DKK1

GO:0051056	regulation of small GTPase mediated signal transduction	-2.52	APOE,TGM2,SLIT2
GO:0050890	cognition	-2.43	APOE,PPT1,DKK1
GO:0000902	cell morphogenesis	-2.37	ADAM8,NEO1,SLIT2,DKK1
GO:0031346	positive regulation of cell projection organization	-2.29	APOE,GRN,SLIT2
GO:1902903	regulation of supramolecular fiber organization	-2.18	APOE,CCN2,SLIT2
GO:0043254	regulation of protein-containing complex assembly	-2.09	APOE,SLIT2,DKK1
WP3888	VEGFA-VEGFR2 signaling	-5.44	CALR,CCN2,IGFBP3,IGFBP7,DKK1,FJX1
GO:0071675	regulation of mononuclear cell migration	-5.16	ADAM8,CALR,THBS1,SLIT2
GO:0009725	response to hormone	-5.07	CALR,CTSB,CTSL,IGFBP7,THBS1,TIMP2,SLIT2
GO:0040017	positive regulation of locomotion	-4.58	ADAM8,BMP4,CALR,GRN,THBS1,SLIT2
GO:1901654	response to ketone	-4.31	CALR,IGFBP7,THBS1,SLIT2
GO:0002685	regulation of leukocyte migration	-4.12	ADAM8,CALR,THBS1,SLIT2
GO:0032870	cellular response to hormone stimulus	-3.92	CALR,CTSB,CTSL,IGFBP7,SLIT2
GO:0048545	response to steroid hormone	-3.82	CALR,IGFBP7,THBS1,SLIT2
GO:0032103	positive regulation of response to external stimulus	-3.70	ADAM8,CALR,GRN,THBS1,SLIT2
GO:0030335	positive regulation of cell migration	-3.63	ADAM8,BMP4,CALR,GRN,THBS1
GO:0002688	regulation of leukocyte chemotaxis	-3.58	CALR,THBS1,SLIT2
GO:2000147	positive regulation of cell motility	-3.55	ADAM8,BMP4,CALR,GRN,THBS1
GO:0010595	positive regulation of endothelial cell migration	-3.54	CALR,GRN,THBS1
GO:0050921	positive regulation of chemotaxis	-3.40	CALR,THBS1,SLIT2
GO:0002687	positive regulation of leukocyte migration	-3.37	ADAM8,CALR,THBS1
GO:0010634	positive regulation of epithelial cell migration	-3.16	CALR,GRN,THBS1
GO:0050920	regulation of chemotaxis	-2.81	CALR,THBS1,SLIT2
GO:0001503	ossification	-5.07	BMP4,CCN2,IGFBP3,PTH1H,MRC2
GO:0001649	osteoblast differentiation	-4.95	BMP4,IGFBP3,PTH1H,MRC2
GO:0032330	regulation of chondrocyte differentiation	-4.70	BMP4,CCN2,PTH1H
GO:0061035	regulation of cartilage development	-4.30	BMP4,CCN2,PTH1H
WP2840	Hair follicle development: cytodifferentiation - part 3 of 3	-4.06	BMP4,CCN2,DKK1
GO:0008544	epidermis development	-3.50	BMP4,CCN2,PTH1H,DKK1
GO:0001501	skeletal system development	-2.85	BMP4,CCN2,PTH1H,TGM2
GO:0043277	apoptotic cell clearance	-5.04	LRP1,TGM2,THBS1
GO:0010466	negative regulation of peptidase activity	-3.47	LRP1,THBS1,TIMP2
GO:0006909	phagocytosis	-3.29	LRP1,TGM2,THBS1

GO:0044092	negative regulation of molecular function	-3.01	APOE,LRP1,THBS1,TIMP2,DKK1
GO:0043086	negative regulation of catalytic activity	-2.91	APOE,LRP1,THBS1,TIMP2
GO:0051346	negative regulation of hydrolase activity	-2.89	LRP1,THBS1,TIMP2
GO:0045861	negative regulation of proteolysis	-2.80	LRP1,THBS1,TIMP2
GO:1902074	response to salt	-2.25	CALR,TGM2,THBS1
WP4747	Netrin-UNC5B signaling pathway	-4.73	CCN2,ITGB4,NEO1
GO:0080135	regulation of cellular response to stress	-3.37	BMP4,CCN2,GRN,NEO1,BCAP31
GO:0007565	female pregnancy	-4.62	CTSB,IGFBP7,ITGB4,PTHLH
GO:0044703	multi-organism reproductive process	-4.43	CTSB,IGFBP7,ITGB4,PTHLH
GO:0044706	multi-multicellular organism process	-4.35	CTSB,IGFBP7,ITGB4,PTHLH
GO:0001707	mesoderm formation	-4.32	BMP4,ITGB4,DKK1
WP2572	Primary focal segmental glomerulosclerosis (FSGS)	-4.30	CTSL,ITGB4,DKK1
GO:0048332	mesoderm morphogenesis	-4.28	BMP4,ITGB4,DKK1
WP5322	CKAP4 signaling pathway map	-3.69	CCN2,ITGB4,DKK1
GO:0048729	tissue morphogenesis	-3.66	BMP4,ITGB4,TGM2,SLIT2,DKK1
GO:0001704	formation of primary germ layer	-3.64	BMP4,ITGB4,DKK1
GO:0030510	regulation of BMP signaling pathway	-3.63	BMP4,NEO1,DKK1
GO:0007498	mesoderm development	-3.56	BMP4,ITGB4,DKK1
GO:0007369	gastrulation	-3.21	BMP4,ITGB4,DKK1
GO:0048736	appendage development	-3.11	BMP4,ITGB4,DKK1
GO:0060173	limb development	-3.11	BMP4,ITGB4,DKK1
GO:0045664	regulation of neuron differentiation	-3.01	BMP4,CALR,DKK1
GO:0051146	striated muscle cell differentiation	-2.89	BMP4,CALR,NEO1
GO:0042692	muscle cell differentiation	-2.57	BMP4,CALR,NEO1
GO:0045596	negative regulation of cell differentiation	-2.27	BMP4,CALR,PTHLH,DKK1
WP5087	Pleural mesothelioma	-2.03	CCN2,ITGB4,DKK1
GO:0046903	secretion	-3.81	NEO1,NMB,PPT1,TGM2,LTBP4
GO:0032940	secretion by cell	-3.16	NEO1,PPT1,TGM2,LTBP4
GO:0023061	signal release	-3.08	PPT1,TGM2,LTBP4
GO:0140352	export from cell	-2.93	NEO1,PPT1,TGM2,LTBP4

Supplementary table 2: Metascape analysis of top 30 upregulated proteins in hA8 KO MDA-MB-231 cells with conditioned media (hA8 KO+CM) against hA8 KO and hA8 KO+CM+GW cells

Term	Description	LogP	Symbols
R-HSA-381426	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	-16.73	ADAM10,APLP2,BMP4,IGFBP3,IGFBP4,IGFBP6,LTBP1,MMP1,FSTL3,PRSS23
R-HSA-8957275	Post-translational protein phosphorylation	-13.09	ADAM10,APLP2,BMP4,IGFBP3,IGFBP4,LTBP1,FSTL3,PRSS23
R-HSA-1474244	Extracellular matrix organization	-14.66	ADAM8,ADAM10,BMP4,BSG,LOXL2,LTBP1,MMP1,SDC4,TGFB1,TGFB2,THBS1
GO:0043410	positive regulation of MAPK cascade	-10.91	ADAM8,BMP4,CCN2,IGFBP3,IGFBP4,IGFBP6,TGFB1,TGFB2,THBS1,DKK1
GO:0043408	regulation of MAPK cascade	-9.43	ADAM8,BMP4,CCN2,IGFBP3,IGFBP4,IGFBP6,TGFB1,TGFB2,THBS1,DKK1
R-HSA-1566948	Elastic fibre formation	-9.23	BMP4,LOXL2,LTBP1,TGFB1,TGFB2
hsa04350	TGF-beta signaling pathway	-9.10	BMP4,LTBP1,NEO1,TGFB1,TGFB2,THBS1
GO:0010718	positive regulation of epithelial to mesenchymal transition	-8.57	ADAM8,BMP4,LOXL2,TGFB1,TGFB2
GO:0048514	blood vessel morphogenesis	-8.30	ADAM8,BMP4,BSG,CCN2,LOXL2,TGFB1,TGFB2,THBS1
GO:0035239	tube morphogenesis	-8.04	ADAM8,BMP4,BSG,CCN2,LOXL2,SDC4,TGFB1,TGFB2,THBS1
GO:0090092	regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	-7.98	BMP4,LTBP1,NEO1,TGFB1,THBS1,FSTL3,DKK1
GO:0001568	blood vessel development	-7.63	ADAM8,BMP4,BSG,CCN2,LOXL2,TGFB1,TGFB2,THBS1
GO:0001525	angiogenesis	-7.61	ADAM8,BMP4,BSG,CCN2,LOXL2,TGFB1,THBS1
GO:1905207	regulation of cardiocyte differentiation	-7.57	BMP4,TGFB1,TGFB2,DKK1
GO:0001944	vasculature development	-7.51	ADAM8,BMP4,BSG,CCN2,LOXL2,TGFB1,TGFB2,THBS1
GO:0090287	regulation of cellular response to growth factor stimulus	-7.43	BMP4,LTBP1,NEO1,TGFB1,THBS1,FSTL3,DKK1
R-HSA-2129379	Molecules associated with elastic fibres	-7.36	BMP4,LTBP1,TGFB1,TGFB2

GO:0030335	positive regulation of cell migration	-7.32	ADAM8,ADAM10,BMP4,CALR,HBEGF,TGFB1,TGFB2,THBS1
GO:0010717	regulation of epithelial to mesenchymal transition	-7.18	ADAM8,BMP4,LOXL2,TGFB1,TGFB2
GO:2000147	positive regulation of cell motility	-7.17	ADAM8,ADAM10,BMP4,CALR,HBEGF,TGFB1,TGFB2,THBS1
GO:0040017	positive regulation of locomotion	-7.10	ADAM8,ADAM10,BMP4,CALR,HBEGF,TGFB1,TGFB2,THBS1
WP560	TGF-beta receptor signaling	-6.65	BMP4,LTBP1,TGFB1,THBS1
WP4816	TGF-beta receptor signaling in skeletal dysplasias	-6.53	BMP4,LTBP1,TGFB1,THBS1
GO:0002040	sprouting angiogenesis	-6.50	BMP4,LOXL2,TGFB1,THBS1
GO:0048660	regulation of smooth muscle cell proliferation	-6.24	BMP4,HBEGF,IGFBP3,TGFB1,THBS1
GO:0051897	positive regulation of phosphatidylinositol 3-kinase/protein kinase B signal transduction	-6.24	ADAM8,HBEGF,TGFB1,TGFB2,THBS1
GO:0043065	positive regulation of apoptotic process	-6.21	ADAM8,BMP4,GSN,IGFBP3,TGFB1,TGFB2,THBS1
GO:1905209	positive regulation of cardiocyte differentiation	-6.20	BMP4,TGFB1,TGFB2
GO:0010634	positive regulation of epithelial cell migration	-6.18	CALR,HBEGF,TGFB1,TGFB2,THBS1
GO:0003170	heart valve development	-6.15	BMP4,TGFB1,TGFB2,DKK1
M5883	NABA SECRETED FACTORS	-6.11	BMP4,HBEGF,MEGF6,TGFB1,TGFB2,FSTL3
GO:0043068	positive regulation of programmed cell death	-6.09	ADAM8,BMP4,GSN,IGFBP3,TGFB1,TGFB2,THBS1
GO:2000725	regulation of cardiac muscle cell differentiation	-5.92	BMP4,TGFB1,DKK1
GO:0001934	positive regulation of protein phosphorylation	-5.87	ADAM8,BMP4,HBEGF,TGFB1,TGFB2,THBS1,DKK1
hsa05205	Proteoglycans in cancer	-5.85	HBEGF,SDC4,TGFB1,TGFB2,THBS1
GO:0043406	positive regulation of MAP kinase activity	-5.83	ADAM8,TGFB1,THBS1,DKK1
GO:0001843	neural tube closure	-5.79	BMP4,SDC4,TGFB1,TGFB2
GO:0060606	tube closure	-5.77	BMP4,SDC4,TGFB1,TGFB2
GO:0014020	primary neural tube formation	-5.68	BMP4,SDC4,TGFB1,TGFB2
GO:0048661	positive regulation of smooth muscle cell proliferation	-5.64	BMP4,HBEGF,TGFB1,THBS1
GO:0042327	positive regulation of phosphorylation	-5.64	ADAM8,BMP4,HBEGF,TGFB1,TGFB2,THBS1,DKK1
GO:0001841	neural tube formation	-5.55	BMP4,SDC4,TGFB1,TGFB2
GO:0090100	positive regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	-5.54	BMP4,NEO1,TGFB1,THBS1

GO:0060485	mesenchyme development	-5.46	BMP4,LOXL2,TGFB1,TGFB2,DKK1
GO:0051896	regulation of phosphatidylinositol 3-kinase/ protein kinase B signal transduction	-5.45	ADAM8,HBEGF,TGFB1,TGFB2,THBS1
GO:0010562	positive regulation of phosphorus metabolic process	-5.35	ADAM8,BMP4,HBEGF,TGFB1,TGFB2,THBS1,DKK1
GO:0045937	positive regulation of phosphate metabolic process	-5.35	ADAM8,BMP4,HBEGF,TGFB1,TGFB2,THBS1,DKK1
GO:0032874	positive regulation of stress-activated MAPK cascade	-5.26	BMP4,CCN2,IGFBP6,TGFB2
R-HSA-9006936	Signaling by TGFB family members	-5.26	LTBP1,TGFB1,TGFB2,FSTL3
GO:0001838	embryonic epithelial tube formation	-5.25	BMP4,SDC4,TGFB1,TGFB2
GO:0070304	positive regulation of stress-activated protein kinase signaling cascade	-5.23	BMP4,CCN2,IGFBP6,TGFB2
GO:0045860	positive regulation of protein kinase activity	-5.18	ADAM8,TGFB1,TGFB2,THBS1,DKK1
R-HSA-114608	Platelet degranulation	-5.16	APLP2,TGFB1,TGFB2,THBS1
GO:0001666	response to hypoxia	-5.15	ADAM8,LOXL2,TGFB1,TGFB2,THBS1
GO:0072175	epithelial tube formation	-5.14	BMP4,SDC4,TGFB1,TGFB2
GO:0032570	response to progesterone	-5.12	TGFB1,TGFB2,THBS1
R-HSA-76005	Response to elevated platelet cytosolic Ca ²⁺	-5.10	APLP2,TGFB1,TGFB2,THBS1
GO:0010632	regulation of epithelial cell migration	-5.08	CALR,HBEGF,TGFB1,TGFB2,THBS1
GO:0002683	negative regulation of immune system process	-5.08	BMP4,SDC4,TGFB1,TGFB2,THBS1,FSTL3
GO:0036293	response to decreased oxygen levels	-5.03	ADAM8,LOXL2,TGFB1,TGFB2,THBS1
GO:0071902	positive regulation of protein serine/threonine kinase activity	-5.02	ADAM8,TGFB1,THBS1,DKK1
GO:0043405	regulation of MAP kinase activity	-5.00	ADAM8,TGFB1,THBS1,DKK1
GO:0016331	morphogenesis of embryonic epithelium	-4.96	BMP4,SDC4,TGFB1,TGFB2
GO:0035148	tube formation	-4.96	BMP4,SDC4,TGFB1,TGFB2
R-HSA-2173789	TGF-beta receptor signaling activates SMADs	-4.93	LTBP1,TGFB1,TGFB2
WP4535	Envelope proteins and their potential roles in EDMD physiopathology	-4.93	CCN2,TGFB1,TGFB2
GO:1905314	semi-lunar valve development	-4.91	BMP4,TGFB1,TGFB2
WP2848	Pluripotent stem cell differentiation pathway	-4.88	BMP4,TGFB1,DKK1
GO:0048729	tissue morphogenesis	-4.86	BMP4,HBEGF,SDC4,TGFB1,TGFB2,DKK1
GO:0070482	response to oxygen levels	-4.84	ADAM8,LOXL2,TGFB1,TGFB2,THBS1
hsa04390	Hippo signaling pathway	-4.83	BMP4,CCN2,TGFB1,TGFB2

hsa05144	Malaria	-4.82	TGFB1,TGFB2,THBS1
GO:0021915	neural tube development	-4.82	BMP4,SDC4,TGFB1,TGFB2
GO:0033674	positive regulation of kinase activity	-4.81	ADAM8,TGFB1,TGFB2,THBS1,DKK1
GO:0003197	endocardial cushion development	-4.80	BMP4,TGFB2,DKK1
WP2406	Cardiac progenitor differentiation	-4.75	BMP4,TGFB1,DKK1
M286	PID TGFB1 PATHWAY	-4.72	CCN2,TGFB1,TGFB2
GO:0050680	negative regulation of epithelial cell proliferation	-4.66	BMP4,TGFB1,TGFB2,THBS1
GO:0007167	enzyme-linked receptor protein signaling pathway	-4.66	BMP4,CCN2,HBEGF,LTBP1,TGFB1,TGFB2
GO:0030336	negative regulation of cell migration	-4.59	BMP4,CALR,IGFBP3,TGFB1,THBS1
GO:0003179	heart valve morphogenesis	-4.56	BMP4,TGFB1,TGFB2
GO:0043009	chordate embryonic development	-4.56	ADAM10,BMP4,SDC4,TGFB1,TGFB2,DKK1
GO:0032872	regulation of stress-activated MAPK cascade	-4.54	BMP4,CCN2,IGFBP6,TGFB2
GO:0070302	regulation of stress-activated protein kinase signaling cascade	-4.51	BMP4,CCN2,IGFBP6,TGFB2
GO:2000146	negative regulation of cell motility	-4.51	BMP4,CALR,IGFBP3,TGFB1,THBS1
WP3624	Lung fibrosis	-4.50	CCN2,PTX3,TGFB1
GO:0048738	cardiac muscle tissue development	-4.48	BMP4,CALR,TGFB1,TGFB2
GO:0048839	inner ear development	-4.48	ADAM10,BMP4,SDC4,TGFB2
GO:0009792	embryo development ending in birth or egg hatching	-4.48	ADAM10,BMP4,SDC4,TGFB1,TGFB2,DKK1
GO:0007178	transmembrane receptor protein serine/threonine kinase signaling pathway	-4.45	BMP4,LTBP1,TGFB1,TGFB2
GO:0014706	striated muscle tissue development	-4.43	BMP4,CALR,TGFB1,TGFB2
GO:1902893	regulation of miRNA transcription	-4.38	BMP4,TGFB1,TGFB2
GO:0051347	positive regulation of transferase activity	-4.38	ADAM8,TGFB1,TGFB2,THBS1,DKK1
GO:1901654	response to ketone	-4.37	CALR,TGFB1,TGFB2,THBS1
GO:0009611	response to wounding	-4.33	CCN2,HBEGF,SDC4,TGFB1,TGFB2
GO:0040013	negative regulation of locomotion	-4.33	BMP4,CALR,IGFBP3,TGFB1,THBS1
GO:0045661	regulation of myoblast differentiation	-4.33	BMP4,IGFBP3,TGFB1
GO:0051129	negative regulation of cellular component organization	-4.29	BMP4,GSN,NEO1,TGFB1,THBS1,DKK1
GO:0045596	negative regulation of cell differentiation	-4.27	BMP4,CALR,TGFB1,TGFB2,FSTL3,DKK1
GO:0043583	ear development	-4.26	ADAM10,BMP4,SDC4,TGFB2
WP5087	Pleural mesothelioma	-4.25	CCN2,HBEGF,TGFB1,PRSS23,DKK1
WP5205	Clock-controlled autophagy in bone metabolism	-4.21	BMP4,TGFB1,DKK1
GO:0002009	morphogenesis of an epithelium	-4.20	BMP4,HBEGF,SDC4,TGFB1,TGFB2

GO:0002062	chondrocyte differentiation	-4.15	BMP4,CCN2,TGFB1
GO:0001837	epithelial to mesenchymal transition	-4.12	LOXL2,TGFB1,TGFB2
GO:2000628	regulation of miRNA metabolic process	-4.12	BMP4,TGFB1,TGFB2
WP2840	Hair follicle development: cytodifferentiation - part 3 of 3	-4.10	BMP4,CCN2,DKK1
GO:0071363	cellular response to growth factor stimulus	-4.05	BMP4,CCN2,TGFB1,TGFB2,THBS1
GO:0140747	regulation of ncRNA transcription	-4.03	BMP4,TGFB1,TGFB2
GO:0022407	regulation of cell-cell adhesion	-4.02	ADAM8,BMP4,SDC4,TGFB1,FSTL3
R-HSA-170834	Signaling by TGF-beta Receptor Complex	-4.02	LTBP1,TGFB1,TGFB2
hsa05323	Rheumatoid arthritis	-4.02	MMP1,TGFB1,TGFB2
GO:0045859	regulation of protein kinase activity	-3.98	ADAM8,TGFB1,TGFB2,THBS1,DKK1
GO:0051153	regulation of striated muscle cell differentiation	-3.96	BMP4,TGFB1,DKK1
R-HSA-76002	Platelet activation, signaling and aggregation	-3.95	APLP2,TGFB1,TGFB2,THBS1
GO:0051223	regulation of protein transport	-3.95	ADAM8,BMP4,NEO1,TGFB1,TGFB2
GO:0050708	regulation of protein secretion	-3.94	ADAM8,NEO1,TGFB1,TGFB2
GO:0002244	hematopoietic progenitor cell differentiation	-3.92	BMP4,TGFB1,FSTL3
GO:0070848	response to growth factor	-3.92	BMP4,CCN2,TGFB1,TGFB2,THBS1
GO:0022612	gland morphogenesis	-3.91	BMP4,TGFB1,TGFB2
hsa05142	Chagas disease	-3.90	CALR,TGFB1,TGFB2
GO:0048545	response to steroid hormone	-3.88	CALR,TGFB1,TGFB2,THBS1
GO:0070201	regulation of establishment of protein localization	-3.86	ADAM8,BMP4,NEO1,TGFB1,TGFB2
GO:0071900	regulation of protein serine/threonine kinase activity	-3.82	ADAM8,TGFB1,THBS1,DKK1
GO:1903532	positive regulation of secretion by cell	-3.80	ADAM8,SDC4,TGFB1,TGFB2
GO:1903557	positive regulation of tumor necrosis factor superfamily cytokine production	-3.80	ADAM8,TGFB1,THBS1
GO:1903530	regulation of secretion by cell	-3.73	ADAM8,NEO1,SDC4,TGFB1,TGFB2
GO:0007507	heart development	-3.73	BMP4,CALR,TGFB1,TGFB2,DKK1
GO:0007423	sensory organ development	-3.70	ADAM10,BMP4,SDC4,TGFB2,NECTIN3
GO:0030326	embryonic limb morphogenesis	-3.69	BMP4,TGFB2,DKK1
GO:0035113	embryonic appendage morphogenesis	-3.69	BMP4,TGFB2,DKK1
GO:0043549	regulation of kinase activity	-3.68	ADAM8,TGFB1,TGFB2,THBS1,DKK1
GO:0051047	positive regulation of secretion	-3.68	ADAM8,SDC4,TGFB1,TGFB2
GO:0048598	embryonic morphogenesis	-3.67	BMP4,SDC4,TGFB1,TGFB2,DKK1
GO:0060562	epithelial tube morphogenesis	-3.67	BMP4,SDC4,TGFB1,TGFB2
GO:0042060	wound healing	-3.64	HBEGF,SDC4,TGFB1,TGFB2
GO:1902105	regulation of leukocyte differentiation	-3.62	ADAM8,BMP4,TGFB1,FSTL3

GO:0002694	regulation of leukocyte activation	-3.62	ADAM8,BMP4,SDC4,TGFB1,THBS1
GO:0003206	cardiac chamber morphogenesis	-3.62	BMP4,TGFB1,TGFB2
GO:0003231	cardiac ventricle development	-3.62	BMP4,TGFB1,TGFB2
GO:0008544	epidermis development	-3.56	BMP4,CCN2,TGFB2,DKK1
GO:0051046	regulation of secretion	-3.55	ADAM8,NEO1,SDC4,TGFB1,TGFB2
GO:0060537	muscle tissue development	-3.52	BMP4,CALR,TGFB1,TGFB2
WP2884	NRF2 pathway	-3.48	HBEGF,TGFB1,TGFB2
GO:0050714	positive regulation of protein secretion	-3.46	ADAM8,TGFB1,TGFB2
GO:0050865	regulation of cell activation	-3.45	ADAM8,BMP4,SDC4,TGFB1,THBS1
GO:0080135	regulation of cellular response to stress	-3.45	BMP4,CCN2,IGFBP6,NEO1,TGFB2
GO:0035107	appendage morphogenesis	-3.44	BMP4,TGFB2,DKK1
GO:0035108	limb morphogenesis	-3.44	BMP4,TGFB2,DKK1
GO:0051338	regulation of transferase activity	-3.34	ADAM8,TGFB1,TGFB2,THBS1,DKK1
GO:0017015	regulation of transforming growth factor beta receptor signaling pathway	-3.32	LTBP1,TGFB1,THBS1
GO:0051147	regulation of muscle cell differentiation	-3.31	BMP4,TGFB1,DKK1
GO:1903844	regulation of cellular response to transforming growth factor beta stimulus	-3.30	LTBP1,TGFB1,THBS1
GO:0003205	cardiac chamber development	-3.26	BMP4,TGFB1,TGFB2
GO:0050678	regulation of epithelial cell proliferation	-3.23	BMP4,TGFB1,TGFB2,THBS1
GO:0051216	cartilage development	-3.23	BMP4,CCN2,TGFB1
GO:0048762	mesenchymal cell differentiation	-3.21	LOXL2,TGFB1,TGFB2
GO:1903706	regulation of hemopoiesis	-3.19	ADAM8,BMP4,TGFB1,FSTL3
GO:0040007	growth	-3.19	ALCAM,BMP4,PTX3,TGFB2
GO:0048589	developmental growth	-3.19	ALCAM,BMP4,PTX3,TGFB2
hsa05152	Tuberculosis	-3.17	TGFB1,TGFB2,MRC2
GO:0048736	appendage development	-3.16	BMP4,TGFB2,DKK1
GO:0060173	limb development	-3.16	BMP4,TGFB2,DKK1
GO:0009725	response to hormone	-3.12	BSG,CALR,TGFB1,TGFB2,THBS1
GO:1903555	regulation of tumor necrosis factor superfamily cytokine production	-3.10	ADAM8,TGFB1,THBS1
GO:0022408	negative regulation of cell-cell adhesion	-3.01	BMP4,SDC4,TGFB1
GO:1903829	positive regulation of protein localization	-2.98	ADAM8,BMP4,TGFB1,TGFB2
GO:0070374	positive regulation of ERK1 and ERK2 cascade	-2.93	BMP4,CCN2,TGFB1
hsa05166	Human T-cell leukemia virus 1 infection	-2.91	CALR,TGFB1,TGFB2
GO:0001501	skeletal system development	-2.90	BMP4,CCN2,TGFB1,TGFB2
GO:0051249	regulation of lymphocyte activation	-2.88	ADAM8,BMP4,SDC4,TGFB1
GO:0061448	connective tissue development	-2.88	BMP4,CCN2,TGFB1
hsa05200	Pathways in cancer	-2.81	BMP4,MMP1,TGFB1,TGFB2
GO:0006954	inflammatory response	-2.74	ADAM8,PTX3,TGFB1,THBS1

GO:0003007	heart morphogenesis	-2.71	BMP4,TGFB1,TGFB2
GO:0051098	regulation of binding	-2.70	BMP4,TGFB1,DKK1
GO:0048608	reproductive structure development	-2.56	BMP4,PTX3,TGFB2
hsa04060	Cytokine-cytokine receptor interaction	-2.55	BMP4,TGFB1,TGFB2
GO:0001822	kidney development	-2.55	BMP4,SDC4,TGFB2
GO:0061458	reproductive system development	-2.54	BMP4,PTX3,TGFB2
GO:0051222	positive regulation of protein transport	-2.51	ADAM8,TGFB1,TGFB2
GO:0072001	renal system development	-2.51	BMP4,SDC4,TGFB2
GO:0007517	muscle organ development	-2.50	HBEGF,TGFB1,TGFB2
GO:0070372	regulation of ERK1 and ERK2 cascade	-2.49	BMP4,CCN2,TGFB1
WP2882	Nuclear receptors meta-pathway	-2.48	HBEGF,TGFB1,TGFB2
GO:1904951	positive regulation of establishment of protein localization	-2.44	ADAM8,TGFB1,TGFB2
GO:0030097	hemopoiesis	-2.42	BMP4,TGFB1,TGFB2,FSTL3
GO:0043010	camera-type eye development	-2.41	BMP4,TGFB2,NECTIN3
GO:0001654	eye development	-2.25	BMP4,TGFB2,NECTIN3
GO:0002696	positive regulation of leukocyte activation	-2.25	ADAM8,TGFB1,THBS1
GO:0001818	negative regulation of cytokine production	-2.25	TGFB1,TGFB2,THBS1
GO:0050863	regulation of T cell activation	-2.25	ADAM8,BMP4,SDC4
GO:1903037	regulation of leukocyte cell-cell adhesion	-2.24	ADAM8,BMP4,SDC4
GO:0150063	visual system development	-2.24	BMP4,TGFB2,NECTIN3
GO:0048880	sensory system development	-2.22	BMP4,TGFB2,NECTIN3
GO:0050867	positive regulation of cell activation	-2.20	ADAM8,TGFB1,THBS1
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	-2.14	CCN2,HBEGF,TGFB1
GO:0048732	gland development	-2.13	BMP4,TGFB1,TGFB2
GO:0010720	positive regulation of cell development	-2.07	ADAM8,BMP4,TGFB1
GO:0048568	embryonic organ development	-2.05	BMP4,TGFB1,TGFB2
M5885	NABA MATRISOME ASSOCIATED	-11.76	ADAM8,ADAM10,BMP4,HBEGF,M EGF6, LOXL2,MMP1,SDC4,TGFB1,TGF B2,FSTL3, ADAMTS15
GO:0030198	extracellular matrix organization	-9.86	ADAM8,ADAM10,LOXL2,MMP1,P TX3, TGFB1,TGFB2,ADAMTS15
GO:0043062	extracellular structure organization	-9.85	ADAM8,ADAM10,LOXL2,MMP1,P TX3, TGFB1,TGFB2,ADAMTS15
GO:0045229	external encapsulating structure organization	-9.83	ADAM8,ADAM10,LOXL2,MMP1,P TX3, TGFB1,TGFB2,ADAMTS15
GO:0022617	extracellular matrix disassembly	-6.97	ADAM8,ADAM10,MMP1,ADAMTS 15
GO:0022411	cellular component disassembly	-6.27	ADAM8,ADAM10,GSN,MMP1,DK K1, ADAMTS15

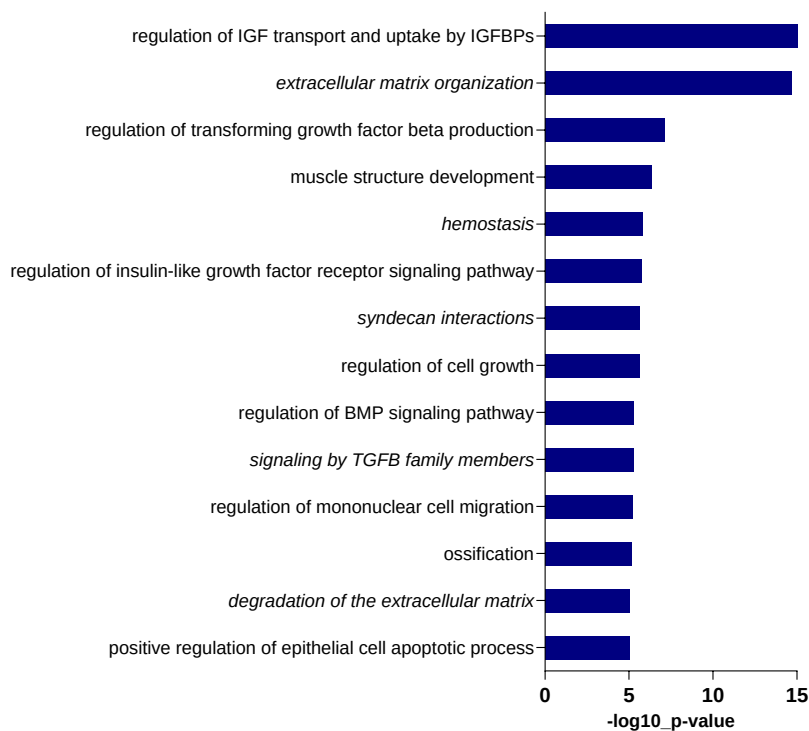
M3468	NABA ECM REGULATORS	-5.54	ADAM8,ADAM10,LOXL2,MMP1,ADAMTS15
R-HSA-1474228	Degradation of the extracellular matrix	-5.02	ADAM8,ADAM10,BSG,MMP1
M3008	NABA ECM GLYCOPROTEINS	-7.55	CCN2,IGFBP3,IGFBP4,IGFBP6,LTBP1,THBS1
M5884	NABA CORE MATRISOME	-6.68	CCN2,IGFBP3,IGFBP4,IGFBP6,LTBP1,THBS1
GO:0043567	regulation of insulin-like growth factor receptor signaling pathway	-5.75	IGFBP3,IGFBP4,IGFBP6
GO:0060828	regulation of canonical Wnt signaling pathway	-3.96	IGFBP4,IGFBP6,TGFB1,DKK1
GO:0030111	regulation of Wnt signaling pathway	-3.54	IGFBP4,IGFBP6,TGFB1,DKK1
GO:0090090	negative regulation of canonical Wnt signaling pathway	-3.49	IGFBP4,IGFBP6,DKK1
WP289	Myometrial relaxation and contraction pathways	-3.35	IGFBP3,IGFBP4,IGFBP6
GO:0030178	negative regulation of Wnt signaling pathway	-3.22	IGFBP4,IGFBP6,DKK1
GO:0006109	regulation of carbohydrate metabolic process	-3.12	IGFBP3,IGFBP4,TGFB1
GO:0071634	regulation of transforming growth factor beta production	-7.13	ADAM8,LTBP1,TGFB2,THBS1
GO:0070613	regulation of protein processing	-4.44	ADAM8,GSN,THBS1
GO:1903317	regulation of protein maturation	-4.37	ADAM8,GSN,THBS1
R-HSA-6798695	Neutrophil degranulation	-2.96	ADAM8,ADAM10,GSN,PTX3
GO:0051346	negative regulation of hydrolase activity	-2.93	PTX3,TGFB2,THBS1
GO:0051336	regulation of hydrolase activity	-2.22	GSN,PTX3,TGFB2,THBS1
GO:0044092	negative regulation of molecular function	-2.19	PTX3,TGFB2,THBS1,DKK1
WP3888	VEGFA-VEGFR2 signaling	-6.87	ADAM10,BSG,CALR,CCN2,HBEGF,IGFBP3,DKK1
GO:0061061	muscle structure development	-6.36	BMP4,CALR,HBEGF,NEO1,TGFB1,TGFB2,ADAMTS15
GO:0051146	striated muscle cell differentiation	-4.28	BMP4,CALR,NEO1,ADAMTS15
GO:0042692	muscle cell differentiation	-3.84	BMP4,CALR,NEO1,ADAMTS15
GO:0035051	cardiocyte differentiation	-3.64	BMP4,CALR,TGFB2
GO:0045664	regulation of neuron differentiation	-3.05	BMP4,CALR,DKK1
R-HSA-109582	Hemostasis	-5.81	APLP2,BSG,MMP1,SDC4,TGFB1,TGFB2,THBS1
R-HSA-202733	Cell surface interactions at the vascular wall	-5.06	BSG,MMP1,SDC4,TGFB1
M198	PID SYNDECAN 1 PATHWAY	-4.93	BSG,MMP1,TGFB1
WP4754	IL-18 signaling pathway	-3.89	BSG,MMP1,PTX3,SDC4
GO:0044089	positive regulation of cellular component biogenesis	-2.89	CCN2,MMP1,SDC4,TGFB1

R-HSA-3000170	Syndecan interactions	-5.64	SDC4,TGFB1,THBS1
GO:0010762	regulation of fibroblast migration	-5.15	SDC4,TGFB1,THBS1
GO:0007162	negative regulation of cell adhesion	-4.96	ADAM10,BMP4,SDC4,TGFB1,THBS1
R-HSA-3000171	Non-integrin membrane-ECM interactions	-4.61	SDC4,TGFB1,THBS1
R-HSA-3781865	Diseases of glycosylation	-3.46	SDC4,THBS1,ADAMTS15
GO:1901888	regulation of cell junction assembly	-2.96	SDC4,THBS1,DKK1
GO:0010810	regulation of cell-substrate adhesion	-2.92	CALR,SDC4,THBS1
R-HSA-5668914	Diseases of metabolism	-2.77	SDC4,THBS1,ADAMTS15
GO:0001558	regulation of cell growth	-5.62	ADAM10,HBEGF,IGFBP3,IGFBP4,TGFB1,TGFB2
GO:0040008	regulation of growth	-4.66	ADAM10,HBEGF,IGFBP3,IGFBP4,TGFB1,TGFB2
GO:0045936	negative regulation of phosphate metabolic process	-3.36	IGFBP3,TGFB1,TGFB2,DKK1
GO:0010563	negative regulation of phosphorus metabolic process	-3.36	IGFBP3,TGFB1,TGFB2,DKK1
hsa04218	Cellular senescence	-3.35	IGFBP3,TGFB1,TGFB2
GO:0030307	positive regulation of cell growth	-3.30	ADAM10,HBEGF,TGFB2
GO:0045216	cell-cell junction organization	-3.17	ADAM10,TGFB1,TGFB2
R-HSA-5663202	Diseases of signal transduction by growth factor receptors and second messengers	-3.13	ADAM10,HBEGF,TGFB1,DKK1
WP2877	Vitamin D receptor pathway	-3.12	IGFBP3,TGFB1,TGFB2
GO:0034330	cell junction organization	-2.83	ADAM10,TGFB1,TGFB2,DKK1
GO:0045927	positive regulation of growth	-2.73	ADAM10,HBEGF,TGFB2
GO:0001933	negative regulation of protein phosphorylation	-2.52	IGFBP3,TGFB1,DKK1
GO:0042326	negative regulation of phosphorylation	-2.43	IGFBP3,TGFB1,DKK1
GO:1901342	regulation of vasculature development	-2.34	ADAM10,TGFB2,THBS1
GO:0031400	negative regulation of protein modification process	-2.08	IGFBP3,TGFB1,DKK1
WP5322	CKAP4 signaling pathway map	-5.35	CCN2,MMP1,TGFB2,DKK1
GO:0030510	regulation of BMP signaling pathway	-5.27	BMP4,NEO1,FSTL3,DKK1
GO:0090288	negative regulation of cellular response to growth factor stimulus	-3.62	THBS1,FSTL3,DKK1
GO:0090101	negative regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	-3.18	LTBP1,FSTL3,DKK1
GO:0071675	regulation of mononuclear cell migration	-5.22	ADAM8,ADAM10,CALR,THBS1
GO:0050921	positive regulation of chemotaxis	-4.96	ADAM10,CALR,TGFB1,THBS1
GO:0002687	positive regulation of leukocyte migration	-4.92	ADAM8,ADAM10,CALR,THBS1

GO:0071677	positive regulation of mononuclear cell migration	-4.33	ADAM8,ADAM10,CALR
GO:0002685	regulation of leukocyte migration	-4.18	ADAM8,ADAM10,CALR,THBS1
GO:0050920	regulation of chemotaxis	-4.16	ADAM10,CALR,TGFB1,THBS1
GO:0002690	positive regulation of leukocyte chemotaxis	-3.97	ADAM10,CALR,THBS1
WP2806	Complement system	-3.96	CALR,PTX3,THBS1
GO:0032103	positive regulation of response to external stimulus	-3.77	ADAM8,ADAM10,CALR,TGFB1,THBS1
GO:0002688	regulation of leukocyte chemotaxis	-3.63	ADAM10,CALR,THBS1
GO:0010595	positive regulation of endothelial cell migration	-3.59	CALR,TGFB1,THBS1
GO:0010038	response to metal ion	-3.44	BSG,CALR,LOXL2,THBS1
hsa04145	Phagosome	-3.39	CALR,THBS1,MRC2
GO:0010594	regulation of endothelial cell migration	-2.86	CALR,TGFB1,THBS1
GO:0010035	response to inorganic substance	-2.83	BSG,CALR,LOXL2,THBS1
GO:0001503	ossification	-5.15	BMP4,CCN2,IGFBP3,MRC2,FSTL3
GO:0032330	regulation of chondrocyte differentiation	-4.75	BMP4,CCN2,LOXL2
GO:0050673	epithelial cell proliferation	-4.71	BMP4,IGFBP3,IGFBP4,LOXL2
GO:0061035	regulation of cartilage development	-4.35	BMP4,CCN2,LOXL2
GO:0001649	osteoblast differentiation	-3.48	BMP4,IGFBP3,MRC2
WP2828	Bladder cancer	-5.12	HBEGF,MMP1,THBS1
hsa05219	Bladder cancer	-5.09	HBEGF,MMP1,THBS1
GO:1903034	regulation of response to wounding	-3.22	HBEGF,NEO1,THBS1
GO:1904037	positive regulation of epithelial cell apoptotic process	-5.02	GSN,TGFB1,THBS1
GO:1904035	regulation of epithelial cell apoptotic process	-3.76	GSN,TGFB1,THBS1
GO:0043254	regulation of protein-containing complex assembly	-3.19	GSN,MMP1,TGFB1,DKK1
GO:1905475	regulation of protein localization to membrane	-3.17	ADAM10,GSN,TGFB1
hsa04514	Cell adhesion molecules	-4.82	ALCAM,NEO1,SDC4,NECTIN3
GO:0031175	neuron projection development	-2.40	ALCAM,APLP2,NEO1,SDC4
GO:0007409	axonogenesis	-2.30	ALCAM,APLP2,NEO1
GO:0061564	axon development	-2.13	ALCAM,APLP2,NEO1
GO:0048667	cell morphogenesis involved in neuron differentiation	-2.08	ALCAM,APLP2,NEO1
GO:0006935	chemotaxis	-4.78	ADAM8,BMP4,BSG,HBEGF,TGFB2
GO:0042330	taxis	-4.77	ADAM8,BMP4,BSG,HBEGF,TGFB2
GO:0040011	locomotion	-4.65	ADAM8,BMP4,BSG,HBEGF,TGFB2
GO:0000902	cell morphogenesis	-4.43	ADAM8,ALCAM,APLP2,NEO1,TGFB2,DKK1
GO:0060326	cell chemotaxis	-4.29	ADAM8,BSG,HBEGF,TGFB2

GO:0045785	positive regulation of cell adhesion	-4.06	ADAM8,CALR,SDC4,TGFB2,FSTL3
GO:0098609	cell-cell adhesion	-3.84	ADAM8,ALCAM,NEO1,TGFB2,NECTIN3
GO:0042476	odontogenesis	-3.69	BMP4,BSG,TGFB2
GO:0030595	leukocyte chemotaxis	-3.42	ADAM8,BSG,TGFB2
GO:0043523	regulation of neuron apoptotic process	-2.85	ADAM8,TGFB2,DKK1
GO:0050900	leukocyte migration	-2.79	ADAM8,BSG,TGFB2
GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	-2.71	ALCAM,TGFB2,NECTIN3
GO:0051235	maintenance of location	-3.22	CALR,GSN,LTBP1
GO:1902905	positive regulation of supramolecular fiber organization	-3.21	CCN2,GSN,SDC4
GO:0051495	positive regulation of cytoskeleton organization	-3.14	CCN2,GSN,SDC4
GO:0110053	regulation of actin filament organization	-2.65	CCN2,GSN,SDC4
GO:0032956	regulation of actin cytoskeleton organization	-2.40	CCN2,GSN,SDC4
GO:0032970	regulation of actin filament-based process	-2.25	CCN2,GSN,SDC4
GO:1902903	regulation of supramolecular fiber organization	-2.22	CCN2,GSN,SDC4

hA8 KO+CM vs hA8 KO, hA8 KO+CM+GW



Supplementary Figure 1: GO annotation and Reactome analysis via Metascape

Reactome and functional enrichment analysis via Metascape for the top 30 significantly abundant peptides in hA8 KO+CM cells in comparison to hA8 KO, hA8 KO+CM+GW. The top 14 enrichment cluster cores are plotted with the p-value. The reactome description is written in italics

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Appendix

I. Abbreviations

ADAM	a disintegrin and metalloprotease
ADAMTS	ADAMs with thrombospondin motifs
ANOVA	analysis of variance
AP	alkaline phosphatase
APP	amyloid precursor protein
APS	ammonium persulphate
AREG	amphiregulin
BCA	bicinchoninic acid
BFA	brefeldin A
BSA	bovine serum albumin
BTC	betacellulin
CCN2	connective tissue growth factor
CM	conditioned media
ConA	concanavalin A
CSF	cerebrospinal fluid
CTD	cytoplasmic domain
CTF	C-terminal fragment
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERAD	ER-associated degradation
EREG	epiregulin
EtOH	ethanol
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G protein-coupled receptor

HA	hemagglutinin
hADAM	human ADAM
HEK	human embryonic kidney
HLA-A	histocompatibility antigen, an alpha chain
IL-6R	interleukin-6 receptor
IRHD	iRhom associated homology domain
iRhom1/2	inactive rhomboid protein 1 and 2
kDa	kilo Dalton
KO	knockout
LPA	lysophosphatidic acid
LPS	lipopolysaccharide
mADAM	murine ADAM
mEF	murine embryonic fibroblasts
MMP	membrane metalloprotease
MPD	membrane-proximal domain
MS	mass spectrometry
MV	microvesicles
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
PFA	paraformaldehyde
PKC	protein kinase C
PM	plasma membrane
PMA	phorbol 12-myristate 13-acetate
PNGase-F	protein N-glycosidase- F
PPC	proprotein convertases
PS	phosphatidylserines
PTM	post-translational modification
rec.	recombinant
RFU	relative fluorescence units
RIP	regulated intramembrane proteolysis
ROS	reactive oxidation species
sADAM	soluble a disintegrin and metalloprotease
sAPP	soluble APP
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA

TAILS	terminal amine isotopic labeling of substrates
TBS	tris-buffered saline
TBS-T	TBS-tween
TCA	trichloroacetic acid
TEMED	tetramethylethandiamin
TFA	trifluoroacetate
TFR	transferrin receptor
TGF- α	tumour growth factor
TGF- β	tumour growth factor- β
TGN	trans-Golgi network
TGN	trans golgi network
THBS1	thrombospondin 1
TM	transmembrane
TMD	transmembrane domain
TNBC	triple-negative breast cancer
TNFR	tumour necrosis factor receptor
TNF- α	tumour necrosis factor- α
WT	wild-type