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Molecular interaction of the human metalloprotease meprin beta with the amyloid precursor protein, ADAM10, and ADAM17 based on proteomics

Dissertation

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Preface

This PhD thesis has been prepared partly cumulative. The data obtained in the presented work has been described in detail in one publication and three manuscripts which are attached as appendix 1, 2, 3, and 4.

1. Metalloprotease Meprin β Generates Nontoxic N-terminal Amyloid Precursor Protein Fragments *in Vivo*.
   Jefferson T*, Čaušević M*, Auf dem Keller U, Schilling O, Isbert S, Geyer R, Maier W, Tschickardt S, Jumpertz T, Weggen S, Bond JS, Overall CM, Pietrzik CU, Becker-Pauly C. August 5, 2011 *The Journal of Biological Chemistry*, 286, 27741-27750.

2. The degradome of the metalloproteases meprin α and β at a glance: TAILS degradomic analysis reveals substrates, inhibitors, and a proteolytic cascade between meprin β and ADAM10.
   Jefferson T, auf dem Keller U, Bellac C, Metz VV, Broder C, Hedrich J, Ohler A, Maier W, Magdolen V, Sterchi EE, Bond JS, Jayakumar A, Traupe H, Pietrzik CU, Postina R, Overall CM, Becker-Pauly C; submitted.

3. The metalloprotease meprin β increases Aβ generation.
   Bien J*, **Jefferson T***, Čaušević M, Isbert S, Postina R, Metz VV, Jumpertz T, Weggen S, Bond JS, Becker-Pauly C, Pietrzik CU; submitted.
   *Shared first authorship

4. The metalloprotease meprin α cleaves the connective tissue growth factor (CTGF) thereby releasing the vascular endothelial growth factor-A (VEGF-A) and enhancing angiogenesis.
   Hedrich J, Nitzsche E, **Jefferson T**, Arnold P, Dejung M, Sterchi EE, Bond JS, Brieger J, Becker-Pauly C; submitted.

In the following, most relevant results from the manuscripts are briefly described and additional data is illustrated in detail. Methods which are not shown in the publication are explained here in the section material and methods.

Mainz, November 2011

Tamara Jefferson

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ABSTRACT

The zinc-endopeptidases meprin α and β are key players in (patho)physiological processes like inflammation, collagen assembly, and angiogenesis. After their discovery in murine brush border membranes and human intestinal epithelia, more expression loci were identified such as leukocytes, cancer cells, and human skin. Animal models, cell cultures, and biochemical assays indicate functions for meprins in epithelial differentiation, cell migration, matrix remodeling, angiogenesis, connective tissue formation, and immunological processes. However, many of their precise physiological substrates remain elusive.

Mass spectrometry based proteomics revealed unique cleavage specificity for acidic amino acid residues in P1´ and identified novel biological substrate candidates. Among 269 extracellular proteins detected in the substrate screen, the amyloid precursor protein (APP) and ADAM10 (a disintegrin and metalloprotease 10) emerged as highly confident candidates. APP is known to have several cleavage sites mediated by different proteases, resulting in diverse implications. Proteolytic processing of one site refers to the β-secretase BACE (β-site APP cleaving enzyme) and is the primary step in the development of Alzheimer´s Disease (AD) since toxic Aβ (amyloid β) peptides are released into the extracellular space aggregating to senile plaques. Membrane-anchored meprin β exhibits β-secretase activity as demonstrated in a cell culture system. In FRET (Fluorescence Resonance Energy Transfer)-based assays, the meprin cleavage efficiency towards Aβ peptides was determined to be 10^4 higher than BACE1 activity. Moreover, meprin β cleaves the first two amino acids (aa), liberating aminoterminally a glutamate residue which then can be cyclized into pyro-glutamate by the human glutaminyl cyclase. The truncated Aβ peptides are specifically generated only in AD patients. Due to a higher hydrophobicity these peptides reveal greater plaque-forming capacity which distributes a greater toxicity. Until now, there was no protease identified processing at this cleavage site. Generation of meprin mediated N-terminal APP fragments was detected *in vitro* and *in vivo.* These N-APP peptides were not toxic to murine and human brain cells although N-APP was previously demonstrated to be a ligand for the death receptor (DR) 6 triggering neuronal degeneration.

ADAM10 is proposed to shed the ectodomain of APP in a non-amyloidogenic pathway. In this regard, we could identify the propeptide of ADAM10 as a substrate for meprin β subsequently leading to an increased activation of ADAM10, demonstrated by FRET analyses, as well as *in vitro* and *in vivo* approaches. Vice versa ADAM10 was shown to shed meprin β into the extracellular space. Shedding was induced either by phorbol 12-myristate 13-acetate (PMA) or the ion carrier A23187, and was blocked by ADAM10 inhibitors. Overall, this thesis revealed a complex proteolytic network in neurophysiology that might be important for the progression of Alzheimer’s Disease.

1 INTRODUCTION

1.1 PROTEASES AND THE PROTEOME

The analysis of protein architecture, interaction, dynamics, and underlying mechanisms in cells are the most challenging goals in biology. Proteases are enzymatic proteins that cleave other proteins by hydrolyzation of their peptide bonds and therewith modify their function. Comprising 1.7% of the whole human genome with 578 representatives points to the importance of this protein class (Puente et al., 2005). All proteins undergo intra- and extracellular posttranslational modifications (PTM) by proteolysis, thus indicating various physiological functions for proteases. Following translation, proteolytic cleavage of the signal peptide is of great importance for the export of the protease into the endoplasmic reticulum that serves for various functions including protein folding. Formation of disulfide bonds between two cysteines in a posttranslational process provides greater stability for the protein. In addition, PTM also involves the control of enzyme activity. Most proteases remain inactive as zymogens until the propeptide is cleaved off.

In the past years, research began to focus on studying the proteome representing the entire proteolytic repertoire including the interaction of substrates and inhibitors (Wilkins et al., 1996). It becomes more essential to identify and discover novel biomarkers and develop drug targets. Today, proteases represent one of the six most important biochemical classes of therapeutics accounting 5-10% alongside with receptors, enzymes, hormones and factors, DNA, nuclear receptors, and ion channels (Drews, 2000). The proteome is in a state of constant change and since proteases cannot operate alone rather are part of a regulated network – the proteolytic web – elucidation of individual proteases´ *in vivo* function is necessary (auf dem Keller et al., 2007; Overall and Kleifeld, 2006). Already small modifications in this proteomic composition can result in a deregulation of proteolysis and cause pathologies in organisms.

Powerful proteomic techniques have been developed to determine the biological role of proteases within their natural context of the proteome. The credibility of protease identification is high since the location of expression (cells, tissues, or organisms), activity status, time-dependent expression levels, and physiological substrates are considered. Among others, two novel tools have been recently introduced into the proteomics field, PICS (Proteomic Identification of protease Cleavage Sites) and (N- and C-) TAILS (Terminal Amine Isotopic Labeling of Substrates), helping to understand the role of proteolysis *in vivo* and entire molecular pathways in the onset and development of diseases (Dean and Overall, 2007; Schilling and Overall, 2008).

Matrix metalloproteases (MMPs) are a large and diverse class of extracellular proteases known to be involved in numerous (patho)physiological processes (Morrison et al., 2009). Using proteomic approaches, the substrate repertoire which defines a protease´s function was expanded and new roles were discovered.

Depending on the active center and underlying catalytic strategies, proteolytic enzymes are classified into seven categories (Barrett, 2001) (http//.merops.sanger.ac.uk): asparagine-, aspartate-, thiol-, glutamate-, serine-, threonine-, and metalloproteases.

1.2 MEPRIN METALLOPROTEASES

Meprin (metalloprotease from renal tissue) α and β are zinc-dependent metalloproteases of the astacin family and belong to the metzincin superfamily (Bode et al., 1993; Stöcker and Bode, 1995). They were first identified in the small intestine and renal tissue of balb/c mice (Sterchi et al., 1982).

Meprins were also found in men when patients who lost their pancreas due to surgery were still capable of cleaving the synthetic chymotryptic substrate N-benzoyl-L-tyrosyl-p-aminobenzoic acid. This compound is used to test pancreatic function, due to the proteolytic activity of chymotrypsin, but was also cleaved by meprins which were then termed PABA peptide hydrolases (PPH) (Sterchi et al., 1982) until they received their today´s effective name meprin (Bond and Beynon, 1986). Subsequently, two differently expressed meprin enzymes were identified, the α- and the β-variant (Gorbea et al., 1991) with highest expression levels in epithelia of intestine and kidney tubules. Further, meprins were localized in the salivary glands (Craig et al., 1991), nose epithelia, smooth muscle cells, and the inner ear (Spencer-Dene et al., 1994). EST (expressed sequence tags) and ISH (*in situ* hybridization) analysis in murine whole mount embryos reveal a broader expression spectrum (www.genepaint.org). Meanwhile, it has been shown that meprins are also expressed under pathological conditions, as in epithelial tumor cells (Matters and Bond, 1999) and colon carcinoma cells where particularly meprin α is secreted apically and basolaterally indicating a role in cancer metastasis (Lottaz et al., 1999). Human keratinocytes as a novel locus have been discovered recently for both meprin proteases (Becker-Pauly et al., 2007). By indirect immunofluorescence, different epidermal layers were verified as expression loci for meprin α and β. Here, meprin α is restricted to the *stratum basale*, whereas meprin β is located in the *stratum granulosum* indicating differential biological roles for the two enzymes.

In humans, rat, and mice meprin exists as two homologous proteins, α and β, sharing an identity of 50% in their amino acid sequence. Meprins (α_1, α_2 and β) were also detected in the zebrafish *Danio rerio* (Schutte et al., 2010; Schutte et al., 2007) demonstrating a quite

ubiquitous expression pattern. However, meprins are exclusively found in vertebrates. Meprin-like astacin metalloproteases, containing MAM domains, were merely identified in the cnidarian *Hydra vulgaris* (Yan et al., 2000), in squid (Kanzawa et al., 2004), and in the horseshoe crab (Becker-Pauly et al., 2009).

Meprins belong to the six human astacins; meprin α and β, BMP-1 (bone morphogenetic protein-1), mTld (mammalian tolloid, a splice variant of BMP-1), Tll-1 and Tll-2 (tolloid like-1 and -2) and ovastacin. All astacins contain the zinc-binding motif (HExxHxxGxxHxxxRxDR) within the protease domain required for catalysis through stabilization of the water molecule by the zinc ion which is complexed by three histidines, a glutamate, and a tyrosine residue (Stöcker and Bode, 1995; Stöcker et al., 1993). Further, metzincins are characterized by a highly conserved methionine residue in a β-1.4 loop (met-turn, SxMHY) building a "hydrophobic pillow" (Gomis-Ruth, 2009; Stöcker and Bode, 1995; Stöcker et al., 1993).

Encoded on two different genes, meprin α on chromosome 6 and meprin β on chromosome 18 in men, the proteases exhibit distinct hallmarks among the astacins. Meprins are complex glycosylated type 1 membrane proteins with a multidomain structure. The proteases are expressed N-terminally with a hydrophobic signal peptide directing the protein to the ER (endoplasmic reticulum)-lumen during biosynthesis. The N-terminal activity regulating propeptide (pro) is followed by the MAM (meprin A5 protein, receptor tyrosine phosphatase μ)- and the TRAF (tumor necrosis factor receptor associated factor)- domain, both involved in protein-protein-interaction. The domain structures of meprin α and β differ mainly in the presence of the 56 aa long inserted (I) domain within meprin α, containing a furin-like cleavage site. During the secretory pathway, proteolytic processing within the I-domain results in the loss of the C-terminal part. Release of the EGF (epidermal growth factor)-, the transmembrane (TM)-, and the cytoplasmic (C) domain results in secretion of the protease in the extracellular space and loss of the membrane anchor. The cytoplasmic domain reaching into the intracellular space was described to be responsible for the transport of newly synthesized meprin (Hengst and Bond, 2004). On the other hand, meprin β lacking the inserted domain remains predominantly bound to the cell surface (Eldering et al., 1997). ADAM (a disintegrin and metalloprotease)17/TACE (tumor necrosis factor-α converting enzyme) has been identified to regulate the shedding of meprin β due to potential N- and O-glycosylation sites which is induced by the phorbol ester PMA (phorbol 12-myristate 13-acetate) (Hahn et al., 2003).

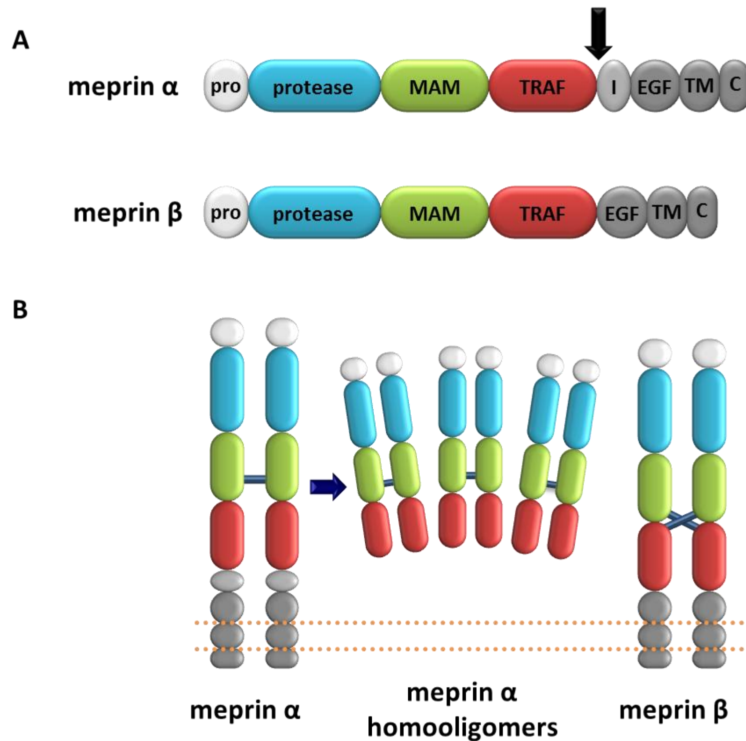


Figure 1: Domain structure and oligomerization of meprin α and β.
(**A**) The black arrow indicates the cleavage site for furin-like convertases in meprin α. Pro: propeptide; protease: astacin-like catalytic domain; MAM: meprin A5 protein, receptor tyrosine phosphatase μ-domain; TRAF: tumor necrosis factor receptor associated factor-domain; I: inserted domain; EGF: epidermal growth factor-like domain; TM: transmembrane domain; C: cytoplasmic domain. (**B**) Intramolecular disulfide bonds (blue bars) are responsible for dimerization of individual subunits. Non-covalent interaction leads to generation of meprin α homooligomers.

Both meprins occur predominantly as homooligomers associated by disulfide bonds due to cysteine residues in the MAM and TRAF domain, respectively, and build larger complexes when further associated by non-covalent connections (Figure 1) (Becker et al., 2003; Bertenshaw et al., 2003; Ishmael et al., 2005). That way, α-homooligomers can build multimers reaching a molar mass up to six mega Dalton forming the largest secreted protease known. Further, rodent meprin was described to connect to α/β heterooligomers which can organize to tetramers (Becker et al., 2003; Bertenshaw et al., 2003; Ishmael et al., 2001).

As most members of the astacin family, meprins are synthesized as inactive proenzymes which impedes the generation of a stabilizing N-terminal salt bridge (Bode et al., 1992). Activation was demonstrated to be operated by different serine proteases. *In vitro,* trypsin, a digestive enzyme of the small intestine, activates both meprins. In colorectal cancer cells the tryptic protease plasmin cleaves meprin α but not β (Becker et al., 2003; Rosmann et al., 2002) which was also evidenced for the neutrophil elastase (Bergin et al., 2008). Recently, particular kallikrein-related peptidases (KLK) were identified as potent activators in human skin. KLK5 activates meprin α and meprin β, whereas the latter is additionally processed by the action of KLK4 and KLK8 (Becker-Pauly et al., 2007; Ohler et al., 2010). Since

proteolysis underlies strong regulation mechanisms, proteases not only need to be activated but are also inhibited under certain conditions preventing or inducing pathologies. The best-known and potent inhibitor for meprin α and β is the hydroxamate actinonin (Kruse et al., 2004; Wang et al., 2010). Fetuin-A and cystatin-C, broad spectrum inhibitors, were also confirmed to endogenously inhibit meprins in blood plasma (Hedrich et al., 2010; Jefferson et al., submitted).

Based on proteomics techniques involving peptide libraries and native substrates, cleavage specificities of meprin α and β were determined (Becker-Pauly et al., 2011; Jefferson et al., submitted). Both proteases favor the acidic amino acids aspartate and glutamate in P1' (nomenclature by Schechter and Berger, 1967) although having a different substrate spectrum. Beside various *in vitro* substrates, to date only a few biological relevant proteins are known to be processed *in vivo* by meprins, such as TGF-α (tumor growth factor α) (Bergin et al., 2008), VEGF-A (vascular endothelial growth factor-A) (Schutte et al., 2010), interleukin (Il)-1β, and 18 (Banerjee and Bond, 2008; Herzog et al., 2009).

Although meprins have been of particular interest over the past decades, quantitative proteomics has only now provided a broad insight into the biology of the metalloproteases offering novel functions by addressing their substrate degradome.

1.3 ALZHEIMER ASSOCIATED PROTEINS APP AND ADAM10

Alzheimer´s Disease is a progressive disorder destroying neurons and causing dementia. Two forms of AD are known, the late-onset sporadic type and the early onset familial Alzheimer´s Disease (FAD). The latter affects primarily younger people and exhibits entirely autosomal dominant inheritance (Selkoe, 2005). Pathogenic mutations have been identified in the genes encoding APP (amyloid precursor protein) and presenelin (PSEN1, PSEN2) causing an increased γ-secretase activity (Cruts M, Rademakers R Alzheimer disease & frontotemporal dementia mutation database. http://www.molgen.ua.ac.be/ ADMutations/).

For several years the transmembrane type 1 protein APP has been described to be crucial for AD. The APP gene is located on chromosome 21 (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). Three exons (7, 8 and 15) can be alternatively spliced into several isoforms (Kang et al., 1987; Ponte et al., 1988; Yoshikai et al., 1990) leading to three major translation products: APP770 and APP751, both containing a Kunitz protease inhibitor (KPI) domain (Kitaguchi et al., 1988; Tanzi et al., 1988), and APP695 which is prevalently expressed in the brain, lacking the inhibitor domain (Figure 2).

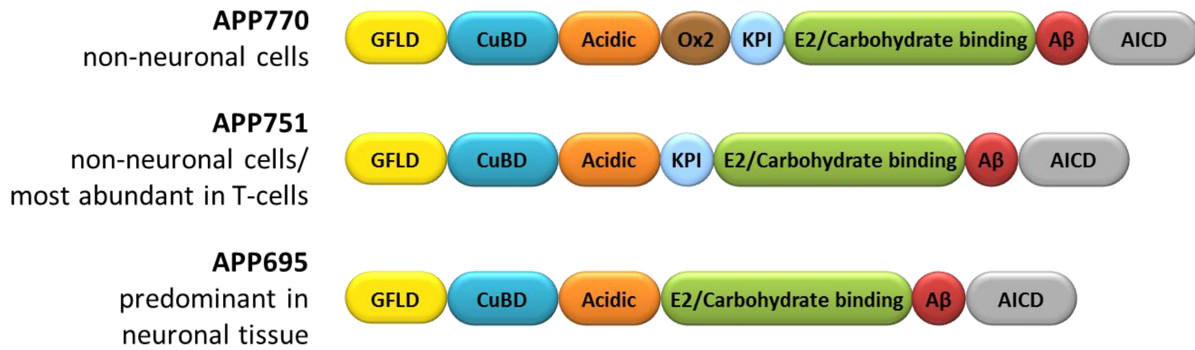


Figure 2: Domain structure of the most prominent APP isoforms.
The full length protein APP770 contains the Ox2 (Orexin receptor type 2) domain which is missing in APP751 and 695. APP695 lacks additionally the KPI domain (Kunitz Protease Inhibitor domain). GFLD: N-terminal growth factor-like domain; CuBD: copper-binding domain; E2: conserved region of the central APP domain; Aβ: amyloid β peptide; AICD: APP intracellular domain.

Under pathological conditions, APP is shed within the extracellular domain by the β-secretase BACE1 (β-site APP cleaving enzyme) generating a soluble s (soluble) APPβ product and a membrane bound C-terminal fragment, termed C99 (Figure 3) (De Strooper and Annaert, 2000). The Aβ (38-42 aa) peptide and an APP intracellular domain (AICD) are released by subsequent γ-secretase (complex composed of presenilin in the catalytic center) processing of C99 (De Strooper and Annaert, 2000). Two major Aβ variants have been detected in human brain. While the shorter $Aβ_{40}$ fragment is the most prominent form, the long-tailed $Aβ_{42}$ comes in lower ratios but is more toxic due to higher accumulation susceptibility. Both peptides are components of senile plaques exhibiting pathological effects on neurons indicating that toxicity might depend on the $Aβ_{40}/Aβ_{42}$ ratio. These cleavage events are described as the “amyloid hypothesis” (Selkoe, 2001).

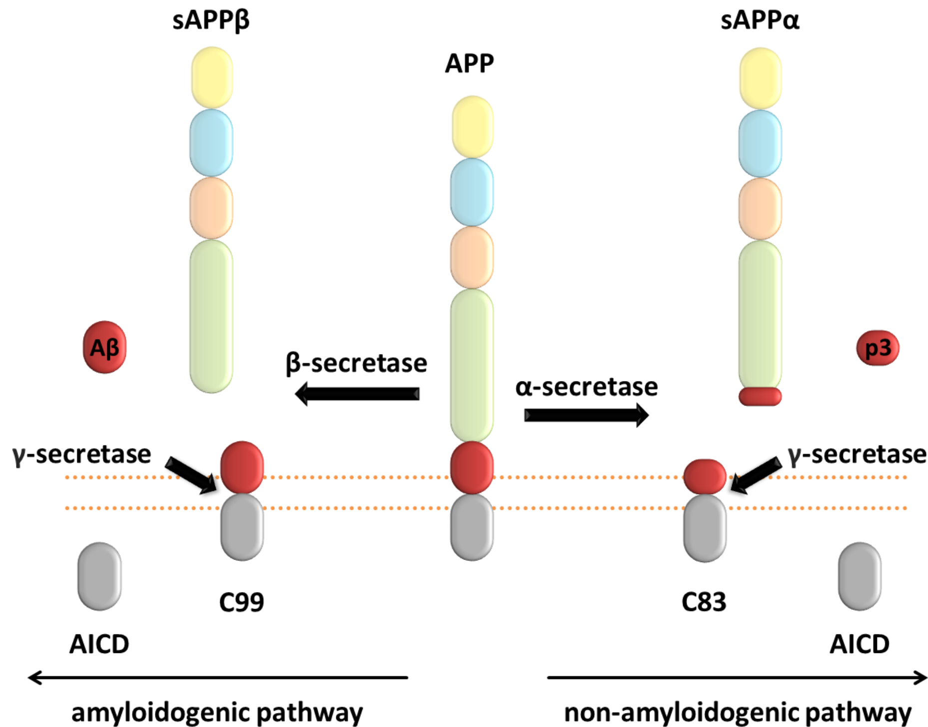


Figure 3: Processing of APP (demonstrated for APP695)
The α-, β- and γ-secretases are involved in APP cleavage. After disruption of the Aβ domain (red) by the α-secretase in the non-amyloidogenic pathway, soluble APPα is released. The remaining membrane bound C83 is further cleaved by the γ-secretase liberating p3 into the extracellular space and an intracellular domain (AICD). In the amyloidogenic pathway, β-secretase activity yields in sAPPβ release. Cleavage by the γ-secretase causes subsequently toxic Aβ peptides and the release of AICD originating from C99. Figure according to (Van Dam and De Deyn, 2006).

In normal physiology, α-secretases process APP within the Aβ site, thereby releasing the sAPPα ectodomain into the extracellular space, preventing generation of Aβ and sAPPβ, subsequently exhibiting neuro-protective properties (Meziane et al., 1998; Stein et al., 2004). The second product after α-secretase cleavage, C83, is additionally fragmented by the γ-secretase into the intracellular AICD50 and the extracellular p3 peptide. The latter is aminoterminally truncated and lacks the first 16 aa of the Aβ peptide. Nevertheless, it was described to be non-toxic (Dulin et al., 2007).

Since APP is a ubiquitously expressed protein with an unknown biological function, involvement in other physiological processes than the generation of AD is obvious. This emphasizes the relevance to take a closer look at the key players of the non-pathological pathway.

Based on *in vitro* cleavage events and overexpression studies that result in sAPPα enrichment, several α-secretase candidates have been suggested. Among others, the metalloproteases ADAM9, 10, and, 17/TACE were the most likely applicants (Freese et al., 2009; Lammich et al., 1999; Slack et al., 2001; Taylor et al., 2009). Recently, knockdown studies in murine cells revealed ADAM10 as the physiologically relevant α-secretase (Kuhn

et al., 2010) consistent with an increased Aβ deposition in mouse brains when catalytically inactive ADAM10 was expressed (Postina et al., 2004). On the other hand, it was reported that primary neurons of conditional ADAM10 knockout mice exhibited a disturbed processing of total APP (Jorisson 2010). Not only sAPPα levels were reduced but also sAPPβ, CTF (C-terminal fragment), and Aβ peptide amounts were lowered.

ADAM10 is a 750 aa transmembrane type 1 protease and belongs to the metzincins. It consists N-terminally of a prodomain, the zinc binding metalloproteinase and disintegrin-like domain. The latter is responsible for interaction between ADAMs and integrins through a disintegrin loop (White, 2003). Followed by the EGF-like domain (Reddy et al., 2000) - also termed cysteine rich domain (Janes et al., 2005) -, the protease is anchored in the membrane by a transmembrane region and a cytoplasmic tail (Figure 4). ADAM10 can be liberated itself from the cell membrane by shedding events mediated by ADAM9 and 15 (Parkin and Harris, 2009).

Figure 4: Domain structure of ADAM10.
Pro: propeptide; EGF: epidermal growth factor, TM: transmembrane domain; C: cytoplasmic tail.

Beside the activity of ADAM10 in the non-pathological processing of APP, the protease cleaves additional substrates such as N-cadherin (Reiss et al., 2005), Ephrin A5 (Janes et al., 2005), Klotho (Chen et al., 2007), and pro-betacellulin (Sahin et al., 2004). Moreover, ADAM10 has been identified as a sheddase for the cell surface receptor Notch1 after ligand binding in embryonic development (Pan and Rubin, 1997).

Regarding APP, the processing mediated by ADAM10 not only alters the ratio between sAPPβ and sAPPα but also expands the biological functions. Yet it is unknown if and how the APP processing products influence each other (Zheng and Koo, 2006). Although the biological role for APP remains elusive, it has been attributed to positively affect cell viability. For instance, APP was reported to promote cell proliferation and differentiation, it was linked to anti-apoptotic effects in neurons and might be involved in calcium metabolism (Copanaki et al., 2010; Meng et al., 2001). Still, all proposed functions need to be proven *in vivo.*

2 AIMS OF THE STUDY

Aβ deposition and senile plaques are the characteristic histopathological hallmarks of Alzheimer's Disease. However, it is still unknown whether these abnormalities cause neuronal damage and lead to progressive dementia.

Cell culture based proteomics revealed the amyloid precursor protein (APP) and the sheddase ADAM10 (a disintegrin and metalloproteinase 10) as putative substrates for meprin β *in vivo.* Although meprins have been studied for decades, only few biological substrates are known and therewith the physiological function for meprins remains elusive.

This thesis focuses the functional role of meprin β in Alzheimer´s Disease and in the network with interacting proteins.

The implication of meprin β activity in APP processing was analyzed in cell culture-based assays, *in vitro,* and *in vivo* studies. An alternative mechanism to the Aβ-dependent neurodegeneration was recently published describing APP binding to DR6, triggering a caspase-dependent axon-pruning pathway (Nikolaev et al., 2009).

i) Meprin β mediated APP fragments were therefore studied to be potentially involved in the DR6 binding process.

ii) ADAM10 induced shedding of meprin β was analyzed in cell culture to reveal the interaction of both proteins.

iii) Moreover, activity regulation of ADAM10 by meprin β and vice versa shedding of meprin β was studied by FRET substrates *in vitro* and *in cellulo.*

ADAM10 as a key player in non-pathological APP cleavage was identified as a substrate for meprin β processing within the propeptide. As for the majority of metalloproteases, the removal of the propeptide is required to gain full activity. Nevertheless, it is documented that the propeptide of ADAM10 owns great inhibition potential (Moss et al., 2007). After initial cleavage by proprotein convertases the ADAM10 propeptide is non-covalently bound to the mature protease as known for ADAM12 (Wewer et al., 2006).

A potential networking of meprin β, APP and ADAM10/17 studied in this thesis may support knowledge in the neurological processes causing Aβ plaques and Alzheimer´s Disease.

3 MATERIAL AND METHODS

3.1 GENERATION OF RECOMBINANT PROADAM17 USING THE BAC-TO-BAC® EXPRESSION SYSTEM

A truncated version of murine proADAM17 was generated consisting of 460 aa and lacking the ADAM17 signal peptide, the transmembrane domain, and the intracellular part by using the following primers:

sense: 5´-CATG**CCATGG**CTGGCACCCCGACCTCCGGAGGAAGCAGGCTCTGGCTCCCATCCGCGAC-3´

antisense: 5´-CCG**CTCGAG**TGCTAACTAGTTGCCACACACCTTGTTGCTGCGCTCCTGGAAGCACTCT-3´

Bold sequences represent restriction sites for *Nco*I and *Xho*I, respectively.

Constructs were ligated into pFASTBAC™1 (Gibco) containing the meprin β signal peptide, followed by a 6 x His-tag, resulting in the expression of soluble proADAM17. Primers were synthesized by Invitrogen GmbH and sequences of constructs were verified by DNA sequencing (Genterprise GmbH). Recombinant protein was expressed using the Bac-to-Bac® expression system (Invitrogen) following the manufacturer's instructions. All media and supplements were obtained from Gibco.

Since generation of proADAM17 and cloning into the expression plasmid pFASTBAC™1 (Gibco) was carried out by Wladislaw Maier (Institute for Pathobiochemistry, University Medical Center at the University Mainz) and insertion of proADAM17 pFASTBAC™1 into baculoviruses was performed by Birgit Schättler (Institute for Cell and Matrix Biology, University Mainz), the procedure of the Bac-to-Bac® expression system (Invitrogen) is shortly described in Figure 5.

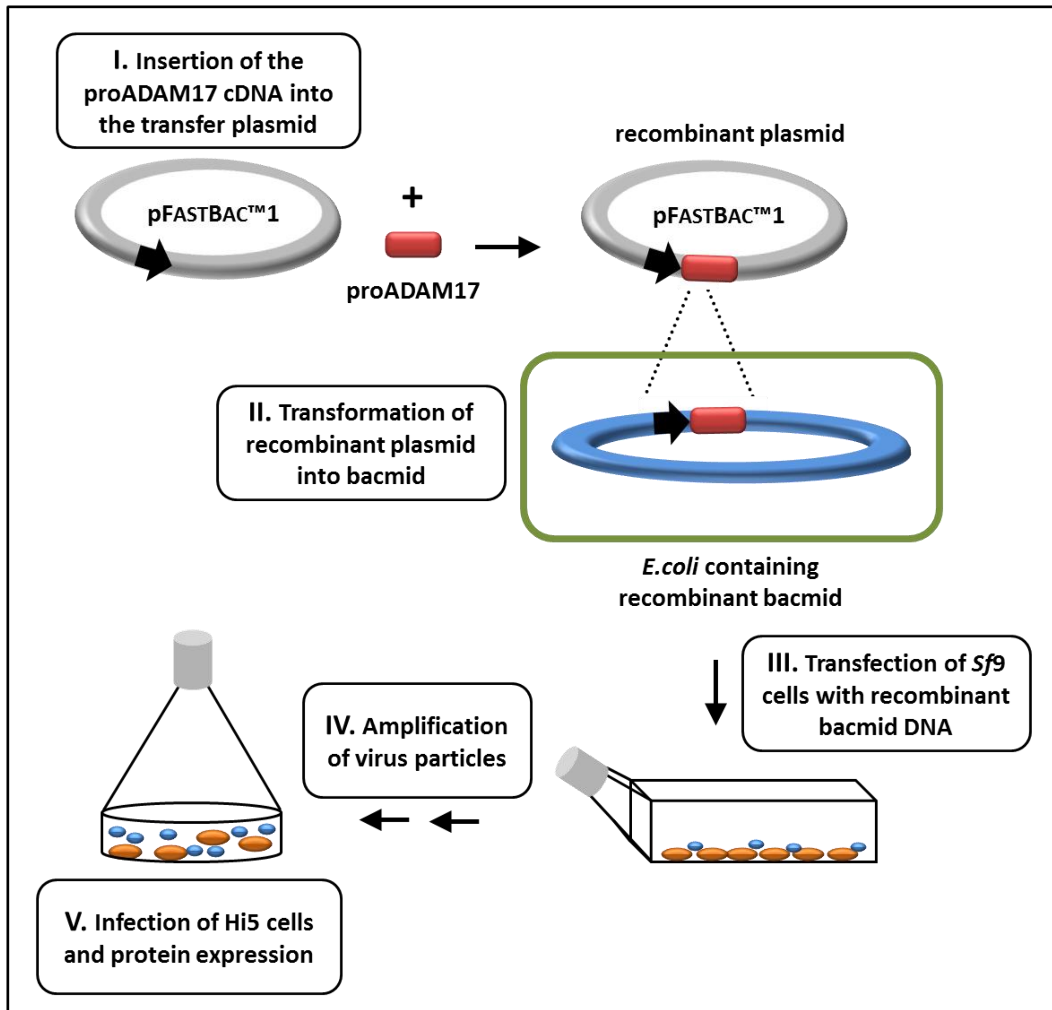


Figure 5: Overview of the baculovirus expression system according to the Bac-to-Bac® system.
I. The expression vector pFASTBAC™1 (grey) contains a gentamycin promotor for subsequent selection, an expression cassette flanked by TN7 recognizing procaryotic transposons and a multiple cloning site for cDNA insertion using the baculovirus specific Polyhedrin promotor (all indicated by the big black arrow). After cloning the target gene (red) into the vector, the recombinant plasmid is inserted into the lac-Z region of *E.coli* DH10 Bac-cells (Invitrogen) containing bacmids (**II.**) which facilitate integration by a helper plasmid encoding for the transposase. Blue-white screening allows further selection of positive transformants. **III+IV**. Following isolation of bacmids, *Sf*9 insect cells are transfected to boost the quantity of baculoviruses. **V.** Hi5 cells are then infected with those to express recombinant proteins on a large scale.

Expression and purification of proADAM17 was performed as described for proADAM10 in the manuscript “The degradome of the metalloproteases meprin α and β at a glance: TAILS degradomics analysis reveals substrates, inhibitors, and a proteolytic cascade between meprin β and ADAM10” (Jefferson et al., submitted). ProADAM17 was analyzed by SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis) and Western blot analysis (monoclonal anti-Penta His 1:1.000, Qiagen; polyclonal anti-ADAM17, 1:1.000, Abcam). For N-terminal sequencing, proteins were blotted onto PVDF membranes, stained with Coomassie Brilliant Blue, and sequenced at the protein micro-sequencing centre of the Institut Fédératif de Recherche (IFR) 128 (Lyon, France).

3.2 PROCESSING OF THE RECOMBINANT PROTEINS ADAM17 AND BACE1 BY MEPRIN β

Recombinant ADAM17 was incubated with meprin β for given periods of time at 37°C in a 250:1 ratio. 1 µg human BACE1 (R&D systems) was processed with 300 ng pro and active meprin β. Proteins were analyzed by SDS-PAGE and Western blot using a polyclonal anti-ADAM17 (Abcam) and a polyclonal anti-BACE1 antibody (provided by Prof. Dr. Claus Pietrzik, Institute for Pathobiochemistry, University Medical Center at the University Mainz).

3.3 TRANSFECTION OF HEK293 CELLS WITH PROADAM17

Cell culture work was done under a culture hood (LaminAir Sterilbank, Holten, Allerød/Dänemark) preventing microbial contamination. Before use, all media and supplements were heated at 37°C, all purchased from Invitrogen, D-Karlsruhe.
Hek (human embryonic kidney) 293 cells were grown in monolayers in 6-well plates under optimal conditions (37°C, 5% CO_2, 95% relative humidity). Medium change took place every three days using Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), and 5% fetal calf serum (FCS).
Transfection is a method allowing the insertion of heterologous cDNA into eukaryotic cells. These were treated with a transfection reagent based on nanoparticles (Nanofectin, PAA). Nanofectin consists of porose nanoparticles enveloped by cationic polymers enlarging the surface and facilitate to bind high DNA quantities which are negatively charged. After binding of polymer-DNA complexes to the cell membrane, they are incorporated endocytotically into the cell. Prior to every transfection, old medium was removed and exchanged by 1 ml fresh full medium per well. Cells were grown to 60-80% confluence and transfected according to the manufacturer´s instructions (Nanofectin, PAA). Soluble ADAM17 was detected with an anti-ADAM17 antibody (Abcam). Signal detection in all Western blot experiments was carried out with the enhanced chemiluminescence (ECL) assay solutions (Millipore).

3.4 PROTEOLYTIC ACTIVITY ASSAY WITH AZOCASEIN

A chemically modified substrate is used to generally determine the activity of proteases (Wolz, 1994) (Figure 6).

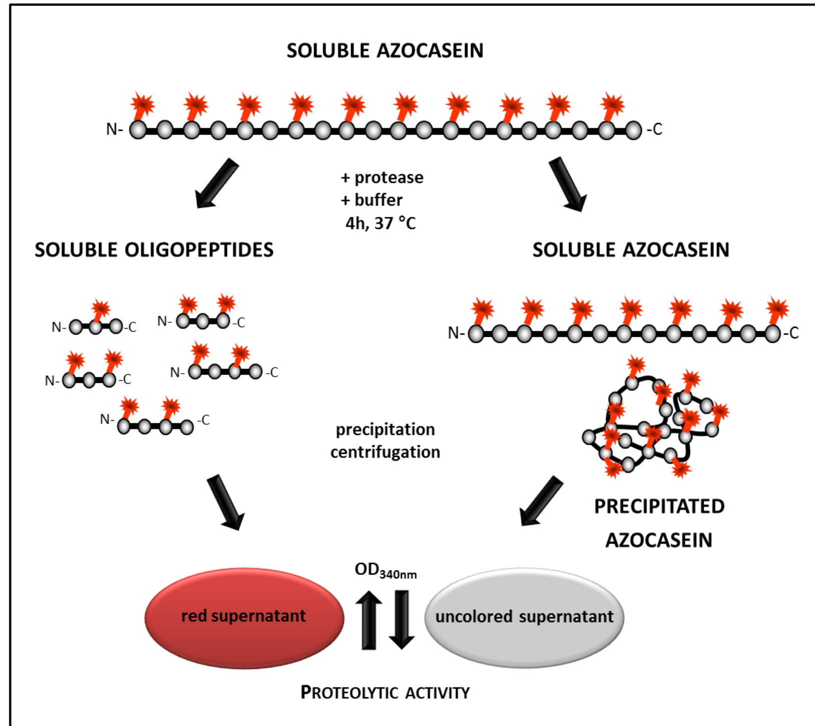


Figure 6: Azocasein assay to determine the proteolytic activity of ADAM10 and 17.
Hydrolysis of the protease substrate azocasein results in peptides of different sizes. Oligopeptide fragments cannot be precipitated and are therefore detectable in the solution. Figure according to Microbial Biofilm Group, Institute of Plant Biology/Microbiology, University of Zürich.

Sulfanilamide groups are covalently bound to the milk protein casein conferring the substrate its reddish color. Degradation of the protein results in liberation of reddish peptide fragments which dissolve in the medium. Hydrolysis proportional to protease activity is subsequently detectable at 340 nm. 5 nM ADAM10 respectively ADAM17 were either measured alone or activated by 0.1 nM meprin β. H_2O served as blank value and 0.1 nM meprin β as positive control. After incubation at 37°C for 2h, each approach was pooled with 200 µl azocasein stock solution (Table 1) and incubated at 37°C for 4h. The proteolytic activity was stopped by adding 600 µl TCA (trichloroacetic acid) and macromolecules were precipitated. Following a 5 min centrifugation at 13.000 x g hydrolyzed azocasein fragments were sedimented. Small peptides and free amino acids cleaved by the proteases remained in solution. The higher the proteolytic activity, the higher the quantity of small peptides and amino acids is in solution, corresponding to a more intense coloring. This was measured at a wavelength of 340 nm.

Table 1: Reagents for the azocasein assay

Reagents	Composition
ethanolamine buffer	20 mM ethanolamine 140 mM NaCl, pH 9.5
azocasein solution	11 mg/ml in ethanolamine buffer

3.5 NATIVE ZYMOGRAPHY

A gelatin zymography enables detection of proteolytic activity due to substrate degradation. After the gelatin substrate is polymerized into the gel, proteases move in the electric field under non-reducing conditions remaining their native structure. Contrary to conventional SDS-PAGE, the native zymography lacks SDS, DTT (dithiothreitol), and heating, preventing protein denaturation (Table 2). 0.5 µM ADAM10 respectively 17 not activated and activated by meprin β at 37°C for 5h were analyzed. Before application to the gel, native 2 x sample buffer was added to each sample. Following the run at 120 V for 4h in native electrophoresis buffer (Table 3), the gel was incubated in a buffer containing 50 mM Tris/HCL, pH 7.5 overnight at 37°C, subsequently fixed in 12% TCA for 1h, and colored with Coomassie solution (Table 4).

Table 2: Composition of native zymography gels

Reagents	7.5% gel
dH_2O	3.6 µl
native buffer (1.5 M Tris, pH 10)	2 µl
acrylamide (30%)	2 µl
TEMED	60 µl
APS	60 µl
gelatin	0.1%

Table 3: Composition of running buffer

Native electrophoresis buffer
25 mM Tris
192 mM Glycin
pH 10

Table 4: Composition of the Coomassie solution

Reagents	Composition
aluminium sulfate hydrate	5%
dH_2O	83%
ethanol	10%
Coomassie Brilliant Blue-G250	0.02%
ortho-phosphoric acid	2%

3.6 FRET ANALYSIS

FRET-based analysis enables the detection of protease activity by hydrolyzation of fluorogenic peptides. Measurements were performed at 37°C in 96-well plates using the fluorescent spectrometer Varioskan Flash (Thermo Scientific). Enzymes were buffered in 50 mM HEPES pH 7.0 or in 0.1 mM sodium acetate pH 4.0. Here, the FRET substrates used for determination of BACE1 activity detected at 405 nm are summarized in Table 5. The full method is described in the manuscript ”The degradome of the metalloproteases meprin α and β at a glance: TAILS degradomics analysis reveals substrates, inhibitors, and a proteolytic cascade between meprin β and ADAM10” (Jefferson et al., submitted).

Table 5: FRET substrates

Substrate	Sequence
meprin β	Mca-YVADAPK(Dnp)-NH_2
BACE1	Abz-VKMDAE-ED(Dnp)-NH_2

4 RESULTS

4.1 SUMMARY OF PUBLISHED DATA

Since most of the data produced in the present dissertation is published or written as a manuscript, a short summarization of it is given. Unpublished studies are described in detail subsequently.

4.2 SUBSTRATE IDENTIFICATION FOR MEPRIN α AND β BASED ON PROTEOMICS

Although the metalloproteases meprin α and β have been studied for many years, the majority of substrates was analyzed by biochemical *in vitro* experiments (Sterchi et al., 2008). Only few proteins are known to be hydrolyzed by meprins *in vivo*, some exhibiting a physiological relevance, like VEGF-A in angiogenesis (Schutte et al., 2010) or IL-18 in inflammation (Banerjee and Bond, 2008).

The novel mass spectrometry based proteomics approach TAILS (Terminal Amine Isotopic Labeling of Substrates) allows for identification of substrate candidates under native conditions using cell cultures, therewith maintaining the protease´s natural environment and cellular context (Butler and Overall, 2009; Doucet and Overall, 2008; Kleifeld et al., 2010; Overall and Dean, 2006). Samples either positive or negative for the protease of interest exhibit a different proteolytic fragmentation. TAILS uses neo N-termini and lysine side chains of these cleavage products caused by the analyzed protease and others, which are then labeled with amine reactive isotopic tags. Following tryptic digestion, novel N-termini caused by the protease of interest are enriched by negative selection. Novel formed internal and C-terminal tryptic peptides of the N-terminome are removed from the pooled proteomes when they are bound by specific polyglycerol aldehyde polymers. Due to isobaric tags, different masses of the novel N-terminomes can be monitored by mass spectrometry and substrates will then be identified bioinformatically (Figure 7).

TAILS was performed with four human cell cultures. In HaCaT (Human adult low Calcium high Temperature keratinocytes), Hek293 (human embryonic kidney), CaCo-2 (Caucasian colon adenocarcinoma), and U373 (glioblastoma-astrocytoma) cells, a total of 269 extracellular putative substrates were identified.

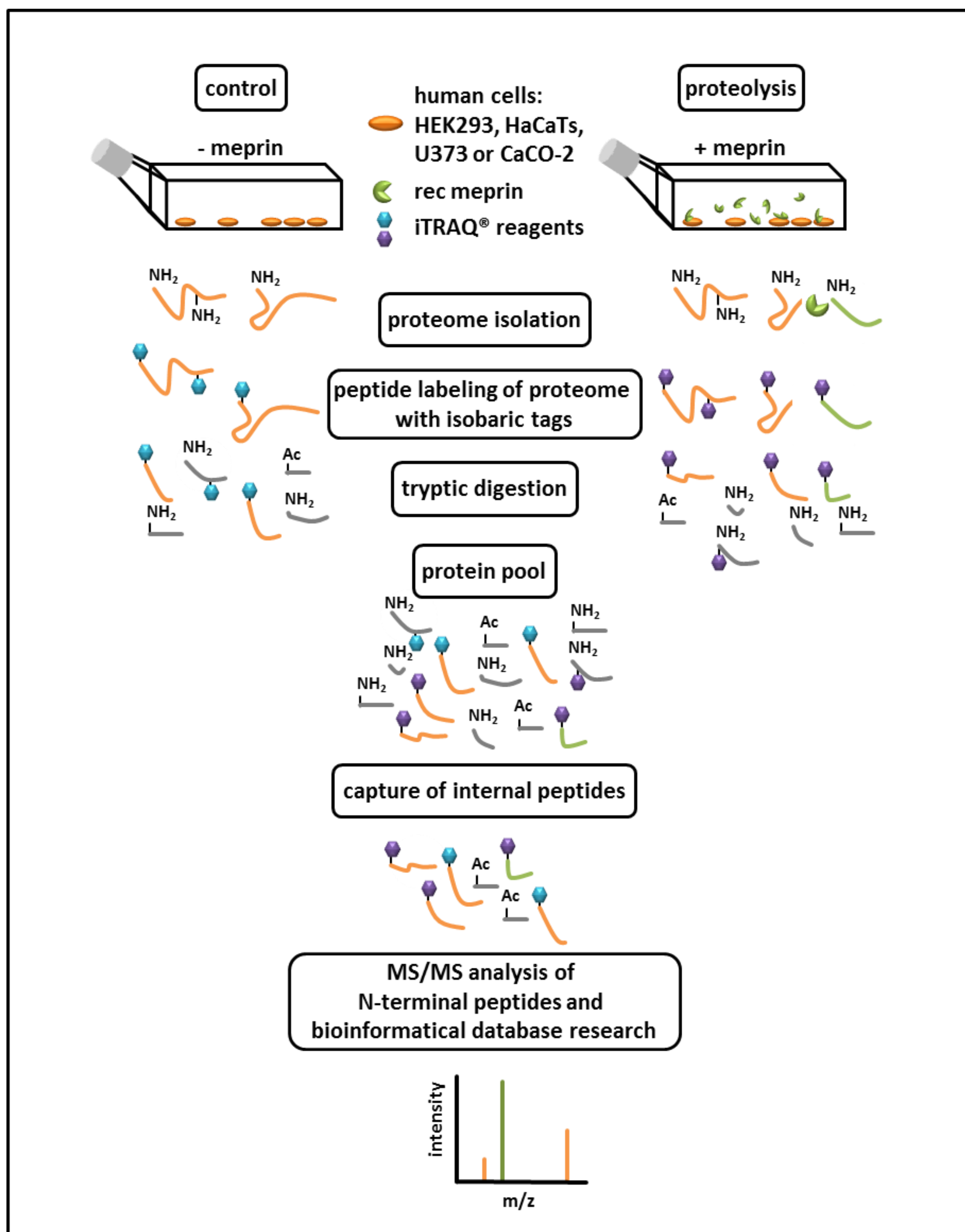


Figure 7: TAILS workflow for identification of meprin substrates.
Novel derived peptides generated by meprin cleavage are illustrated in green with purple iTRAQ® (isobaric tags for relative and absolute quantitation) tags, wildtypical peptides in orange with light blue iTRAQ® tags, and tryptic peptides. Due to different masses of the iTRAQ® reagents, TAILS enables analysis of four samples simultaneously, the scheme illustrates exemplary two.

Belonging to a variety of protein classes - including extracellular matrix proteins, proteases, inhibitors, growth factors, receptors, and hormones - 13 highly confident candidates of those revealing an iTRAQ (isobaric tags for relative and absolute quantitation) ratio >3 were analyzed in follow-up experiments. Moreover, the cleavage specificities of meprins with a preference for aspartate and glutamate in P1´ (Becker-Pauly et al., 2011) have been confirmed by TAILS, further supporting the identity of the substrates.

Figure 8 schematically illustrates a set of identified and biochemically validated substrates within the proteolytic network indicating potential interactions between candidates and meprin α and β.

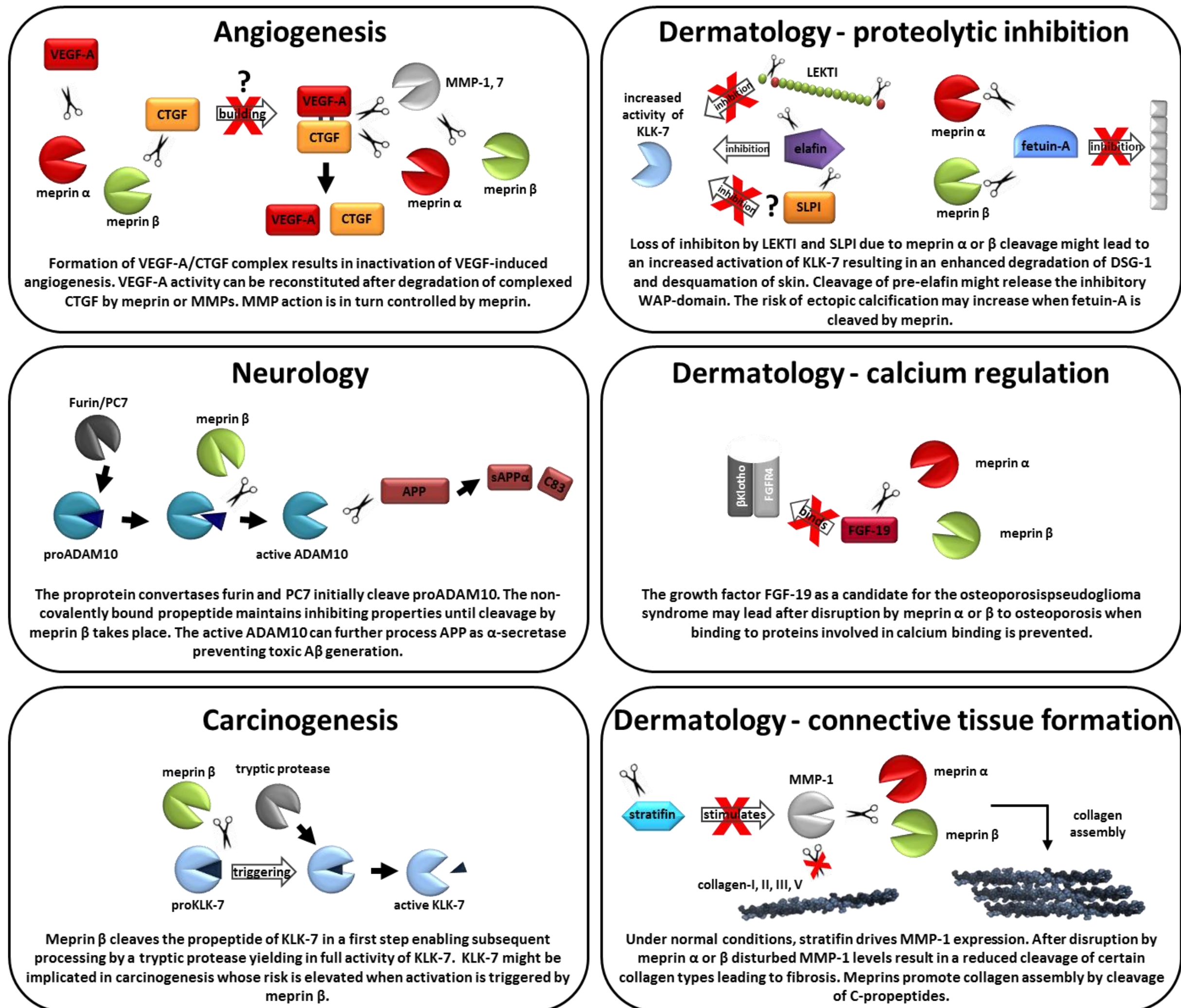


Figure 8: The proteolytic network of meprin α and β.
Meprin α is illustrated in red, meprin β in green. Scissors indicate cleavage. CTGF: connective tissue growth factor; VEGF-A: vascular endothelial growth factor-A; MMP: matrix metalloprotease; LEKTI: Lympho-epithelial Kazal-type-related inhibitor; SLPI: Secretory leukocyte protease inhibitor; KLK: kallikrein; DSG: desmoglein; PC7: proprotein convertase 7; ADAM: a disintegrin and metalloprotease; APP: amyloid precursor protein; FGF: fibroblast growth factor.

4.3 Meprin β activates ADAM10 and is shed into the extracellular space

After successful expression of soluble ADAM10 in insect cells, an activation of ADAM10 was determined due to propeptide disruption mediated by meprin β. Validation was performed *in vitro* by Western blotting using a specific ADAM10 antibody and in FRET analyses (Figure 9A,B). For confirmation, the fluorogenic ADAM specific substrate Mca-KPLGLA2pr(Dnp)AR-NH_2 was analyzed revealing a duplicated activation of ADAM10 after meprin β incubation and

specific ADAM10 inhibition with GI254023X and GM6001 (Figure 9B). Strengthening the putative physiological interaction of meprin β and ADAM10, a reduced ADAM10 activity was observed in murine meprin β deficient fibroblasts (Figure 9C).

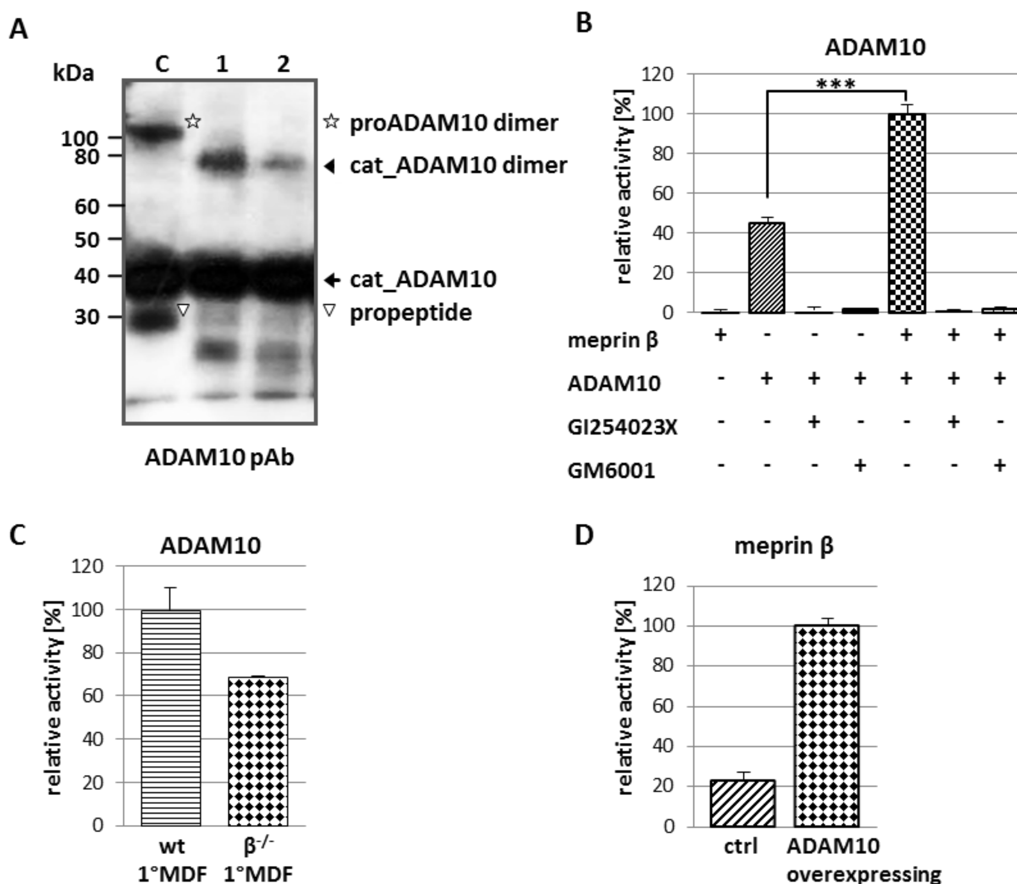


Figure 9: Influence of meprin β on ADAM10 and vice versa of ADAM10 on meprin β.
(**A**) Meprin β processed the 30 kDa propeptide of ADAM10 (triangle) yielding in smaller protein fragments. The proADAM10 dimer (asterisk), consisting of the propeptide and the catalytic domain, decreases accordingly in size after meprin β incubation as shown by the arrowhead which points the dimerized catalytic domain. The arrow indicates cat_ADAM10 comprising the protease domain. (**B**) ADAM10 is activated by meprin β as analyzed by the FRET substrate Mca-KPLGLA2pr(Dnp)AR-NH_2 and can be inhibited by the ADAM inhibitors GI254023X and GM6001. Significance was determined by the t-test ($p<0.01$ ***). (**C**) FRET analysis exhibits that ADAM10 activity decreases to 65% in meprin $\beta^{-/-}$ primary murine dermal fibroblasts (1°MDF). (**D**) ADAM10 overexpressing Hek293 cells result in an increase of meprin β activity when the meprin β specific FRET substrate Mca-YVADAPK(Dnp)-NH_2 is applied. ctrl: control; pAb: polyclonal antibody.

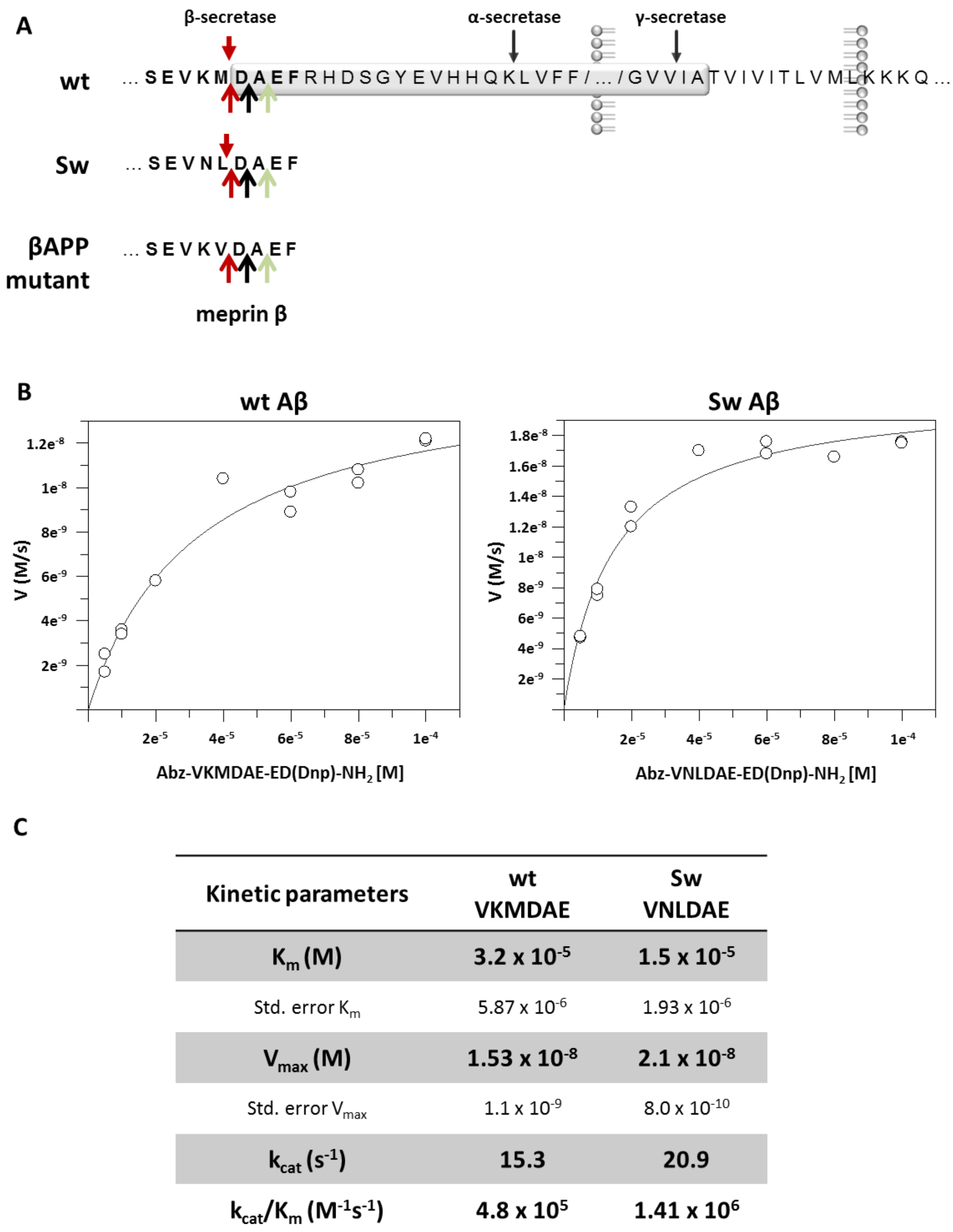
Interestingly, in cell culture ADAM10 sheds meprin β from the cell surface (Jefferson et al., submitted), an event which was formerly contributed to ADAM17/TACE only (Hahn et al., 2003). In cells overexpressing ADAM10, the activity of soluble meprin β increases correspondingly, indicating that meprin β and ADAM10 share a common function in APP processing (Figure 9D).

4.4 THE METALLOPROTEASE MEPRIN β PROCESSES THE AMYLOID PRECURSOR PROTEIN

Cleavage of the Alzheimer´s Disease target protein APP was also determined by the proteomics approach for meprin β. We identified the release of N-terminal APP cleavage products of 11 and 20 kDa molecular mass due to meprin β processing. These proteolytic events were validated *in vitro,* in cell culture based systems, and *in vivo* studying human and mouse brain lysates confirming the biological relevance of the meprin/APP interaction. However, the function of these N-APP products is unknown since they do not effect primary rat neurons, therefore likely no consistence to toxic N-APP DR (death receptor) 6 ligands exists (Nikolaev et al., 2009).

Further, meprin β was identified as a novel enzyme increasing the release of Aβ peptides. Three cleavage sites within APP were determined by mass spectrometry: Asp-672, Ala-673, and Glu-674 (Figure 10A). The latter exposes a glutamate in P1´ allowing potential cyclization due to the glutaminyl cyclase resulting in higher toxicity of Aβ aggregates (Schilling et al., 2008). The first site, Asp-672, is consistent with the BACE1 cleavage site. Analyzing the cleavage efficiency for meprin β towards the wildtypical (wt) APP sequence (Abz-V<u>KM</u>DAE-ED(Dnp)-NH_2) and the mutated sequence in the Swedish variant (SwAPP) (Abz-V<u>NL</u>DAE-ED(Dnp)-NH_2), we determined a meprin activity with a k_{cat}/K_m of $4.8x10^5$ $M^{-1}s^{-1}$ for the wt substrate and $1.4x10^6 M^{-1}s^{-1}$ for the Sw substrate (Figure 10B,C). BACE1 was reported to cleave wildtype APP with a k_{cat}/K_m of 40 $M^{-1}s^{-1}$ respectively 62 $M^{-1}s^{-1}$ (Lin et al., 2000; Shi et al., 2001), the SwAPP 49-118 fold faster (Schechter and Ziv, 2008).

In cell lysates, Aβ formation was increased due to overexpression of soluble meprin β and could be inhibited by a specific metalloprotease inhibitor confirming the biochemical data.



Kinetic parameters	wt VKMDAE	Sw VNLDAE
K_m (M)	3.2×10^{-5}	1.5×10^{-5}
Std. error K_m	5.87×10^{-6}	1.93×10^{-6}
V_{max} (M)	1.53×10^{-8}	2.1×10^{-8}
Std. error V_{max}	1.1×10^{-9}	8.0×10^{-10}
k_{cat} (s^{-1})	15.3	20.9
k_{cat}/K_m ($M^{-1}s^{-1}$)	4.8×10^{5}	1.41×10^{6}

Figure 10: Kinetic analysis of meprin β cleavage efficiency towards APP peptides.
(**A**) Arrows pointing up indicate identified cleavage sites for meprin β by mass spectrometry. The Aβ peptide sequence is marked by the grey box. Down pointing arrows present cleavage sites of the β-, the α-, and the γ-secretases. βAPP mutant is not cleaved by BACE but by meprin β at the same positions as wt and Sw substrates. (**B**) Kinetic analysis of Aβ cleavage following meprin β incubation with the wt FRET substrate Abz-VKMDAEED(Dnp)-NH_2 and the Sw substrate Abz-VNLDAEED(Dnp)-NH_2. Abz: aminobenzoic acid; ED(Dnp): Ethylenediamine 2,4-dinitrophenyl; M: molar mass; s: seconds; v: velocity. (**C**) Kinetic parameters for wt and Sw FRET substrates. Amino acids are given in one letter code. The enzyme concentration used in all assays was 1×10^{-9} M and final substrate concentration ranged from 5 µM to 100 µM.

5 FURTHER RESULTS

5.1 HETEROLOGOUS EXPRESSION AND PURIFICATION OF PROADAM10 AND 17

Shedding of the transmembrane protein meprin β is induced by ADAM17/TACE (Hahn et al., 2003). To analyze whether the inhibition of the catalytic activity of TACE is contributed to the propeptide as reported for ADAM10 (Moss et al., 2007) gave reason to express and purify the protein. Cloning of murine ADAM10 and 17 cDNA into the pFASTBAC™1 plasmid and subsequent insertion into baculoviruses was performed by Wladislaw Maier (Dpt. of Pathobiochemistry, University Medical Center at the University Mainz) and Birgit Schättler (Dpt. of Cell and Matrix Biology, University Mainz), respectively. After transfection of *Sf*9-cells and four-fold amplification to yield a high virus titer, Hi5 cells were infected with corresponding ADAM expressing baculoviruses. An N-terminal His-tag allowed purification of the protein via a Ni-NTA column, while a C-terminal truncation lacking the regions downstream the protease domain assured solubility of the protein. Purification of the recombinant enzyme was proven by MALDI-TOF (data not shown) and Western blot analysis (Figure 11).

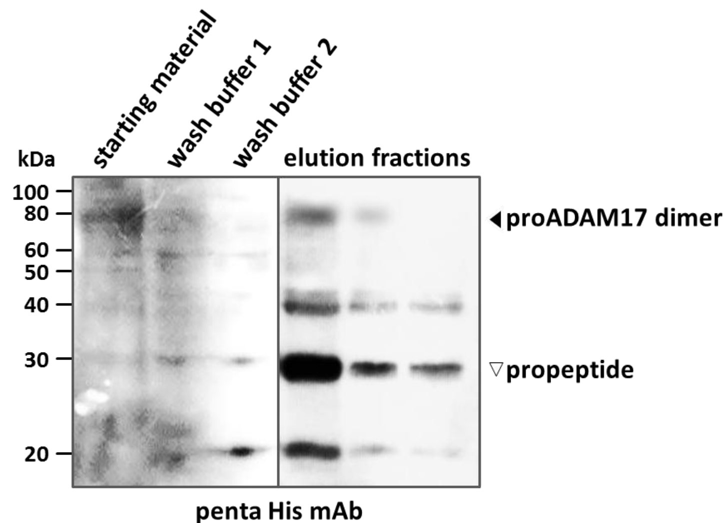


Figure 11: Heterologous expression and purification of soluble ADAM17.
Purified protein was analyzed by a 10% SDS-PAGE and subsequent Western blot analysis. After transferring the proteins to a polyvinylidene fluoride membrane, probing occurred with a monoclonal anti-Penta His (histidine) antibody detecting the N-terminal His-tag.

The protein was eluted using a 50 mM imidazole containing elution buffer. The anti-His antibody determined several ADAM17 species which were identified as the proADAM10 dimer (~90 kDa), comprising the isolated catalytic domain (~40kDa) and the propeptide, and the propeptide (~30 kDa) (Figure 12). Besides an N-terminal degradation product of 20 kDa

size comprising the propeptide as confirmed by MALDI analysis, another 40 kDa signal was detected in the Western blot. This protein species contains only the protease domain as confirmed by mass spectrometry.

5.2 PROTEOLYTIC PROCESSING OF RECOMBINANT ADAM17 BY MEPRIN β *IN VITRO*

To analyze the proteolytic interaction of meprin β and ADAM17, recombinantly expressed protein was incubated for different periods of time with meprin β. Analysis was performed by Western blotting and the anti-His antibody detecting the N-terminus as well as Coomassie staining (Figure 12).

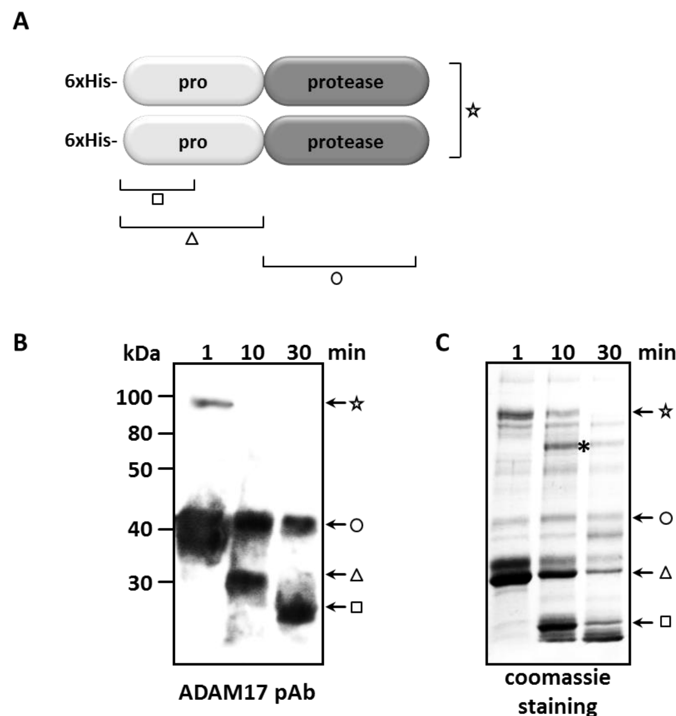


Figure 12: Cleavage of ADAM17 by meprin β. (**A**) Structure of C-terminally truncated ADAM17 with the N-terminal His-tag. Square, triangle, circle, and asterisk present domains as indicated. (**B**) 10% SDS gel followed by Western blotting and anti-Penta His antibody detection revealed cleavage within the propeptide (triangle, square). (**C**) Coomassie staining of 10% SDS gel. The black asterisk shows partially cleaved propeptide of the full length protein. Pro: propeptide; pAb: polyclonal antibody.

The TACE full length protein comprising the dimerized pro- and the protease domain was not detectable anymore in the Western blot after ten minutes of meprin incubation. At the same time, the propeptide decreased in size, indicating proteolytic processing (Figure 12B, triangle, square). The Coomassie stained SDS gel correlated with the proteolytic events observed in the Western blot (Figure 12C). Further, the antibody visualized also processing of the protease domain (Figure 12B, circle). In the gel, meprin β activity led to a signal below

the full length protein which might correspond to the cleaved propeptide (Figure 12C, black asterisk). Prolonged incubation time with meprin resulted in the further processing of this protein species as well as of the propeptide.

5.3 TRANSIENT TRANSFECTION OF HUMAN CELLS WITH ADAM17

To investigate whether meprin β and ADAM17 interact not only *in vitro* but also *in cellulo*, Hek293 cells were transiently transfected with ADAM17 cDNA. Since visualization via SDS gel and specific ADAM17 antibody detection was not feasible, fluorogenic activity assays were performed (Figure 13). ADAM17 activity was measured in a time frame spanning five to 48 hours past initial transfection.

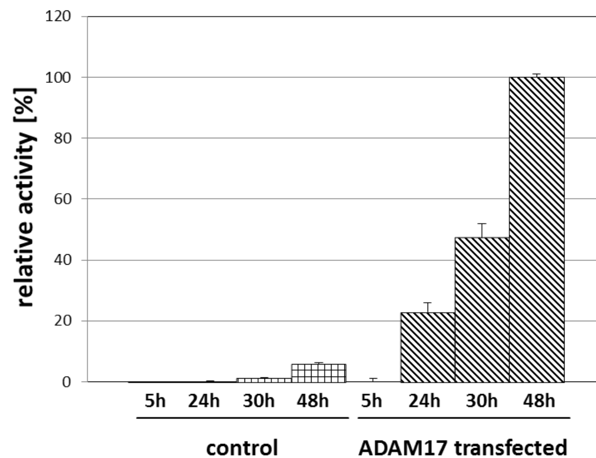


Figure 13: ADAM17 activity in transfected Hek293 cells.
ADAM17 activity was measured using the FRET substrate Mca-KPLGLA2pr(Dnp)AR-NH2 in untreated control and ADAM17 overexpressing Hek293 cells 5h, 24h, 30h and 48h past transfection. h, hours.

The activity of ADAM17 raised parallel to the increasing time with a maximal activity after 48h whereas no activity was detectable 5h post transfection.

5.4 ANALYSIS OF PROTEOLYTIC ACTIVITY OF ADAM10 AND 17

The activity of ADAM10 and 17 induced by meprin β was tested with the artificial substrate azocasein. After hydrolyzation of the substrate by the proteases, the reaction was blocked by trichloroacetic acid. Following removal of precipitates from the solution, the extinction which is proportional to the enzymatic activity of the proteases was measured (Figure 14).

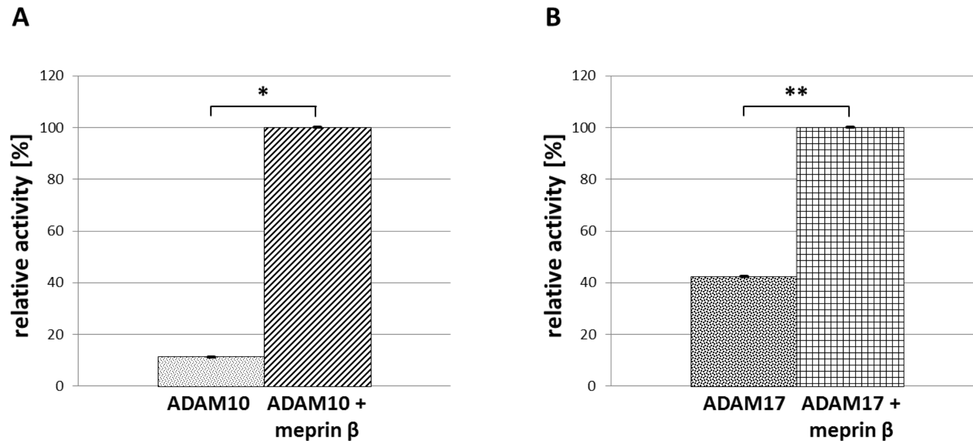


Figure 14: Activity of ADAM10 and 17 is induced by meprin β.
(**A**) ADAM10 activity is about 10-fold increased by meprin β after 2h incubation. (**B**) Following the same incubation time, ADAM17 is twice more active due to meprin β. Significance was determined by the t-test ($p<0.1$*, $p<0.05$ **).

Activity of both recombinant ADAM proteases was significantly increased by meprin β, ten times for ADAM10 twice for ADAM17, using azocasein as a substrate (Figure 14). The activity of both ADAMs was further tested in a native zymography to examine whether ADAM10 and ADAM17 are gelatinolytic enzymes. Whereas TACE activity is only faintly detectable, ADAM10 activity is visualized in the zymography as presented in Figure 15.

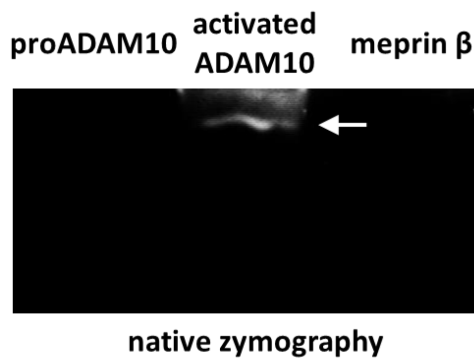


Figure 15: Coomassie-stained zymography gel to determine meprin β-induced activity of ADAM10. ProADAM10 and active meprin β served as controls. Only ADAM10 prior incubated for 5h with meprin β exhibited proteolytic activity in the 10% zymography gel (indicated by the white arrow).

As documented in the native zymography, ADAM10 is not active as a zymogen (Figure 15). Gelatinous activity is specifically gained by prior incubation with meprin β.

5.5 PROCESSING OF BACE1 BY MEPRIN β

The β-secretase BACE1 is reported to be involved in the generation of neurotoxic Aβ peptides. As other aspartic proteases; maturation of proBACE1 implies complex post-translational modifications including the removal of the N-terminal propeptide

(Benjannet et al., 2001). Members of the proprotein convertase family, ubiquitously expressed furin ahead, are believed to be responsible for zymogen processing of proBACE1 between Arg-45 and Glu-46 within the Golgi apparatus (Creemers et al., 2001). Cleavage occurs at RLPR/E, an activation site lacking the typical furin recognition motif with an arginine or lysine residue in P2. Therefore, BACE1 was incubated with zymogenic and active meprin β for different periods of time (Figure 16). Therewith, neutral (pH 7.0) and acidic (pH 4.0) conditions were chosen since BACE1 is an aspartic protease and high activities have been detected in acidic milieus.

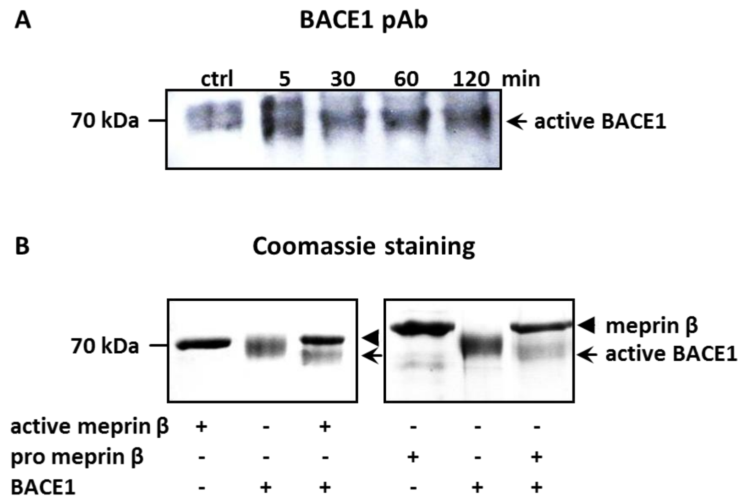


Figure 16: Activation of BACE1 by pro and active meprin β using different pH values.
(**A**) Following incubation of BACE1 with active meprin β at pH 7.0, cleavage products revealing a smaller molecular mass than the full length protein were determined on a 12% SDS gel and subsequent detection with an anti-BACE1 antibody as indicated by the arrow. (**B**) Identical cleavage products were identified on a 12% Coomassie-stained gel when BACE1 was incubated with active meprin β at pH 7.0 (left panel) and pro meprin β at pH 4.0 (right panel). The arrowhead indicates meprin β (zymogenic and active). pAb: polyclonal antibody.

Prolonged incubation of BACE1 with pro meprin β under acidic conditions resulted in an accumulation of active BACE1 indicated by a protein shift (Figure 16A, B right panel). Active meprin β was also capable cleaving BACE1 when present at a neutral pH value (Figure 16B, left panel).

The influence of zymogenic and active meprin β on BACE1 was further investigated by FRET analysis using a fluorogenic substrate that corresponds to the wt APP cleavage site Abz-VKMDAE-ED(Dnp)-NH_2. Since BACE1 is active in acidic intracellular compartments but meprins are present in neutral environments, different pH values have been tested. PH 4.0 and pH 7.0 for BACE1 respectively for meprin β (pro and active) activity alone, pre-incubation of meprin β (pro and active) and BACE1 at pH 4.0, and subsequent measurement at pH 7.0 (pH 4.0→7.0), and vice versa pre-incubation at pH 7.0 and measurement at pH 4.0 (pH 7.0→4.0).

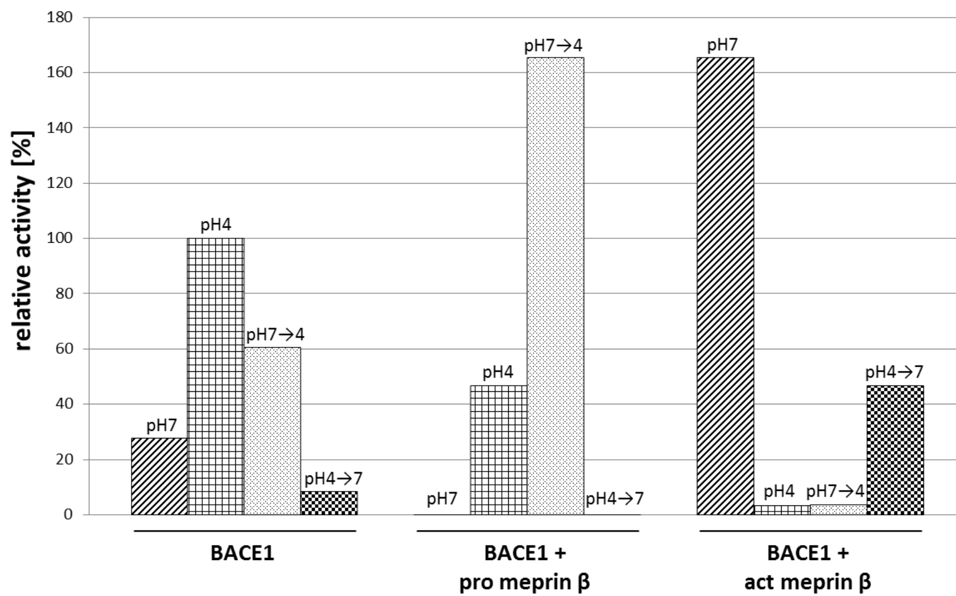


Figure 17: FRET analysis of BACE1 activated by meprin β.
BACE1, BACE1 incubated with zymogenic meprin β (pro meprin β), and BACE1 incubated with active (act) meprin β were analyzed at different pH values as indicated. PH 4.0→7.0 demonstrates that incubation was performed at the pH 4.0 and subsequently measured at pH 7.0 after rebuffering. For pH 7.0→4.0 it was contrary. Meprin β activity (pro and active) was subtracted at particular pH value from every individual approach.

BACE1 was proteolytically active at pH 4.0 with a relative activity of 100% and activity was strongly decreased at pH 7.0 (28%) (Figure 17). Incubation at pH 7.0 with subsequent analysis at pH 4.0 yielded in 60.6%, in the converse set up in 8% activity. When BACE1 was processed by pro meprin β, no BACE activity was detectable at pH 7.0 and pH 4.0→7.0 indicating that the end-point pH value determines BACE1 activity. At pH 7.0→4.0 BACE1 cleavage efficiency was highly increased compared to pH 4.0. This holds also for BACE1 incubated by active meprin β at pH 7.0 and at 4.0→7.0. Although meprin β activity was subtracted from each approach, one cannot exclude that BACE1, working as an enhancer, might lead to an increased activity of meprin β at pH 7.0 since it is very unlikely that the aspartyl protease itself exhibits great proteolytic activity under this condition. BACE1 activity was below 4% at pH 4.0 and 7.0→4.0. The FRET analysis clearly demonstrates that zymogenic and active meprin β both activate BACE1 under certain pH values.

6 DISCUSSION

After publication of more than 50.000 related papers, the amyloid hypothesis is the most convincing to explain the development of Alzheimer´s Disease. Here, soluble Aβ peptides accumulate over time, thereby inducing neurodegenerative disorders in human brain. Around these Aβ peptides, a network consisting of senile plaques, α-, β- and γ-secretases as well as hyper-phosphorylation of intracellular tau proteins has been discovered. However, recent clinical trials and animal studies targeting the key players in this scenario failed to prove the hypothesis (Aisen, 2009; Dodart et al., 2002*)*. Still the function of Aβ and APP is enigmatic primarily because APP knockout mice show little phenotypical differences compared to wildtype counterparts (Heber et al., 2000).

In this work, the link between the generation of fast aggregating Aβ peptides, the generation of N-terminal fragments of APPs, and the regulation of ADAM10 and 17 activity by the metalloprotease meprin β *in vitro* and *in cellulo* has been demonstrated. These results strengthen the role for meprin β as a regulator in the complex network of APP processing.

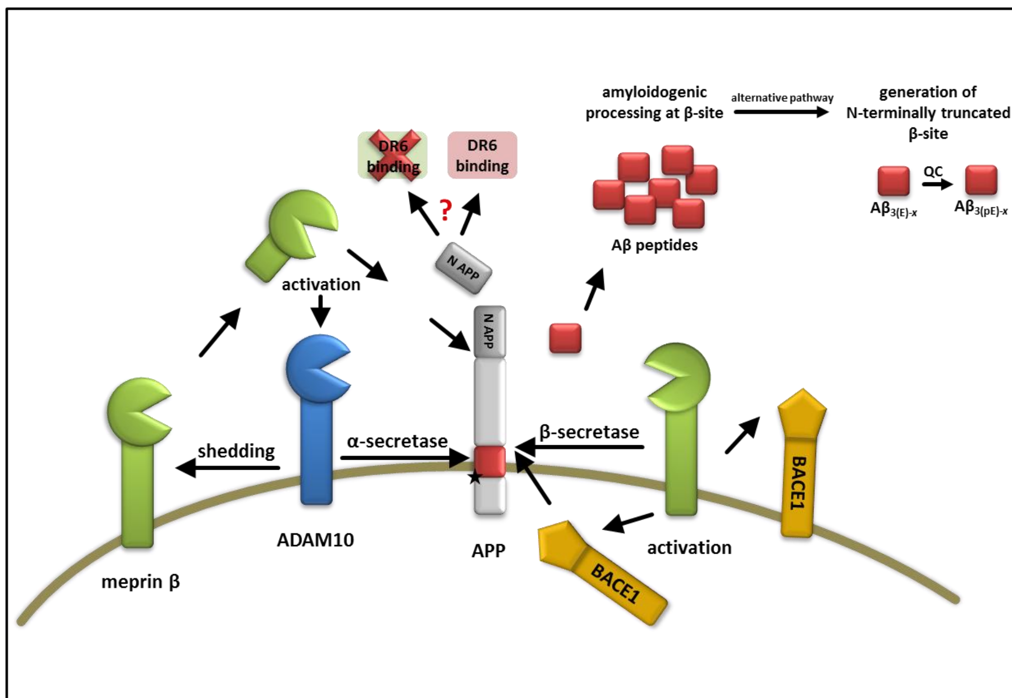


Figure 18: Proteolytic network of APP, meprin β, and other secretases. Membrane-bound meprin β (green) is shed by ADAM10 (blue). Soluble meprin β processes the N-terminus of APP (grey) releasing N-APP which might lead to binding to the death receptor 6 (DR6) or promotes axonal proliferation. Further, meprin activates ADAM10 which in turn cleaves APP as the α-secretase prior to γ-secretase activity (asterisk) resulting in the release of non-toxic sAPPα. APP is processed at the β-site either by membrane bound meprin β or BACE1 (yellow) which was prior activated by meprin β releasing the Aβ peptide (red). Further, liberated glutamate in position 3 of the Aβ peptide ($A\beta_{3(E)-x}$) by meprin β may be cyclized by the glutaminyl cyclase (QC) into pyro-glutamate $A\beta_{3(pE)-x}$.

The aspartic acid BACE1 is widely accepted to be the major β-secretase generating toxic Aβ peptides starting with an N-terminal aspartate residue. Additionally, regarding the low catalytic efficiency towards APP, it might be possible that other proteases are involved in APP processing and Aβ generation. *In cellulo* experiments and kinetic analyses demonstrated that membrane bound meprin β is able to generate Aβ peptides with high efficiency. This is further supported by mass spectrometry based proteomics, revealing striking cleavage specificity for meprin β with a preference for aspartate and glutamate in P1´ (Becker-Pauly et al., 2011). Aβ can then be released from the APP precursor after C-terminal γ-secretase processing, subsequently aggregating to extracellular plaques. Alternatively, releasing the glutamate residue in position 3 of the Aβ peptide ($A\beta_{3(E)\text{-}x}$) could also be contributed to meprin β allowing further cyclization by the glutaminyl cyclase. After generation of a pyroglutamate ($A\beta_{3(pE)\text{-}x}$), Aβ forms higher amyloidogenic aggregates critical for AD development (Schilling et al., 2008).

BACE1 and APP, both transmembrane proteins, were shown to be co-localized in intracellular compartments (Hussain et al., 2000; Vassar et al., 1999). While APP has a short half-time life at the cell membrane, the majority is trafficked through the intracellular secretory and recycling pathways where it encounters BACE1 and the γ-secretase (Sannerud et al., 2011). Intracellular Aβ is most likely generated in the late Golgi apparatus, trans-Golgi networks (TGN), and early endosomes with low pH values (Vassar et al., 2009). Although zymogenic BACE1 was described to process APP to Aβ (Benjannet et al., 2001; Creemers et al., 2001; Huse et al., 2000), mature BACE1 is twice as active as the immature protein (Vassar et al., 2009). The β-secretase cycles between the cell surface and organelles several times (Walter et al., 2001). Intracellular maturation was contributed to furin in the TGN although the cleavage site (RLPR/E) at Glu-46 is rather untypical for furin or furin-like proteases, exhibiting a proline in P2 instead of a positively charged residue (Bennett et al., 2000). Supposedly, immature BACE1 is responsible for the generation of intracellular Aβ peptides (Creemers et al., 2001). Western blot and FRET analyses demonstrated that zymogenic meprin β activates proBACE1 at acidic pH values indicating that other proteases than furin process BACE1. Prior to re-internalization of BACE1, the enzyme could also be activated at the cell surface by mature meprin β consistent with the cleavage preference for glutamate in P1´. Whether extracellular BACE1 activation contributes to Aβ generation or is involved in different processes is speculative.

Under non pathological conditions the majority of extracellular APP is processed by the α-secretase ADAM10 (Jorissen et al., 2010; Kuhn et al., 2010). In recent years, research has focused mainly the generation of Aβ peptides and the development of AD, therefore molecular mechanisms of the regulation of ADAM10 are yet not clear. An ADAM10 knockout in mice induces reduction of the β-secretase activity without altering the β-secretase level

(Jorissen et al., 2010). Otherwise, decreased Aβ is associated with ADAM10 overexpression in transgenic mice (Postina et al., 2004) consistent with an elevated ADAM10 activity and decreased Aβ production when BACE1 is reduced (Vassar et al., 1999). The cause for this contrary data has yet not been revealed indicating that both proteases compete with each other for APP with an unknown molecular biology.

Cell-culture based assays identified ADAM10 as a sheddase releasing the meprin β ectodomain from the cell membrane. Moreover, *in vitro* and *in cellulo* studies demonstrated that ADAM10 activity was increased after meprin β incubation. Initial propeptide removal is mediated by furin or PC7 (proprotein convertase 7) at the furin cleavage site RVKRR (Anders et al., 2001). Not covalently bound to the active protein, the propeptide remains inhibiting the catalytic activity. As a result, mature ADAM10 cleaves APP within the Aβ peptide and releases an extracellular sAPPα fragment preventing the formation of Aβ peptides. ADAM17 which was shown to shed meprin β from the cell surface as well (Hahn et al., 2003) was thought to be an α-secretase candidate until recent studies proved differently (Jorissen et al., 2010; Kuhn et al., 2010). Nevertheless, meprin β activates ADAM17 and ADAM10 in a reminiscent pattern.

A novel model for the development of AD suggested a potential binding of an N-terminal APP (N-APP35) fragment to the death receptor 6 triggering a caspase-dependent pathway leading to axonal pruning causing neurodegenerative diseases like Alzheimer´s Disease (Nikolaev et al., 2009). Mass spectrometry, Western blot analyses, and knockout mice studies identified soluble meprin β as a putative candidate for the generation of N-APP35 which is further fragmented into N-APP20 and N-APP11. Primary neurons were not affected by N-APP peptides querying whether meprin mediated N-APP products have a direct effect on the development of AD. Nevertheless, the N-terminus of APP was shown to increase neurite growth (Small et al., 1994) indicating a potential function for meprin mediated N-APP fragments.

Overall, this work demonstrates that meprin β is involved in the proteolytic network of Alzheimer´s Disease. Nevertheless, contribution of meprin β to this network is not restricted to pathophysiological disorders since all counterparts are expressed under normal conditions.

7 OUTLOOK

To date it has not been demonstrated *in vivo* whether Aβ peptides are the primary cause for Alzheimer´s Disease and how other APP fragments effect the progression of this neurodegenerative pathology. BACE1 remains the major β-secretase initiating Aβ release, although molecular mechanisms are elusive. Primarily, it is necessary to find out by which proteases APP is regulated *in vivo.* This will enable to elucidate underlying mechanisms and to address therapeutic issues. The involvement of meprin β in APP processing confirmed by the data presented in this work and therewith putative involvement in Alzheimer´s Disease could give further insights into the biological role of both, APP and BACE1. Analysis of APP overexpressing mice crossbred with meprin β knockout animals will reveal evidence for the pathophysiological function of meprin β in neurobiology and in Alzheimer's Disease.
Although the amyloid hypothesis has not been proven yet, it appears that it is more complex than initially believed, probably comprising more pathways than amyloidosis. Tau hyper-phosphorylation inducing severe synaptic defects supports the Aβ cascade indicating that a regulation of synaptic structures by the DR6/APP interaction could be part of the amyloid pathway. Whether pruning processes involve N-APP peptides *in vivo* requires further studies and might identify meprin β as the protease releasing the responsible N-APP35 fragment. Specific inhibition of meprin β in neurons and therewith DR6 activation would demonstrate the involvement of meprin β and N-APPs in Alzheimer´s Disease. Protection of neurons by meprin β releasing N-APP11 and -20 fragments was previously demonstrated. Nevertheless, N-APP35 associated with meprin β processing requires further analysis to prove if it might have detrimental activity, too.
Moreover, ADAM10 recently identified as the α-secretase responsible for processing APP *in vivo*, exhibits novel therapeutic potential. Selective activation would be a beneficial function to prevent extracellular Aβ generation. Regarding meprin β as novel candidate for ADAM10 activation facilitates new therapeutic approaches. However, this is to balance on a knife's edge due to the β-secretase activity of meprin β.
Conclusively, mass spectrometry based analysis contributed to the identification of novel physiological substrates of proteolytic enzymes on a large scale. Therewith, the understanding of the biological role of meprins expanded widely, helping to elucidate the physiological function in the proteolytic network.

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9 APPENDIX

9.1 PUBLICATION 1

N-terminal processing of APP by meprin β.

Metalloprotease Meprin β Generates Nontoxic N-terminal Amyloid Precursor Protein Fragments *in Vivo*.

Jefferson T*, Čaušević M*, Auf dem Keller U, Schilling O, Isbert S, Geyer R, Maier W, Tschickardt S, Jumpertz T, Weggen S, Bond JS, Overall CM, Pietrzik CU, Becker-Pauly C. August 5, 2011 The Journal of Biological Chemistry, 286, 27741-27750.

*Shared first authorship

http://www.jbc.org/content/286/31/27741.long

9.2 PUBLICATION 2

The degradome of meprin alpha and beta.

The degradome of the metalloproteases meprin α and β at a glance: TAILS degradomics analysis reveals substrates, inhibitors, and a proteolytic cascade between meprin β and ADAM10.

Jefferson T, auf dem Keller U, Bellac C, Metz VV, Broder C, Hedrich J, Ohler A, Maier W, Magdolen V, Sterchi EE, Bond JS, Jayakumar A, Traupe H, Pietrzik CU, Postina R, Overall CM, Becker-Pauly C; submitted.

9.3 PUBLICATION 3

Meprin β cleaves APP at the β-site.

The metalloprotease meprin β increases Aβ generation.

Bien J*, **Jefferson T***, Čaušević M, Isbert S, Postina R, Metz VV, Jumpertz T, Weggen S, Bond JS, Becker-Pauly C, Pietrzik C; submitted.

*Shared first authorship

9.4 PUBLICATION 4

The metalloprotease meprin α in angiogenesis

The metalloprotease meprin α cleaves the connective tissue growth factor (CTGF) thereby releasing the vascular endothelial growth factor-A (VEGF-A) and enhancing angiogenesis.

Hedrich J, Nitzsche E, **Jefferson T**, Arnold P, Dejung M, Sterchi EE, Bond JS, Brieger J, Becker-Pauly C; submitted.

9.5 ABBREVIATIONS

aa	amino acid
ADAM	a disintegrin and metalloprotease
AICD	APP intracellular domain
APP	amyloid precursor protein
APS	ammonium persulfate
Aβ	amyloid β peptide
BACE	β-site APP cleaving enzyme
BMP1	bone morphogenetic protein-1
C	cytoplasmic
°C	degree celsius
CaCo-2	Caucasian colon adenocarcinoma cells
cDNA	complementary DNA
CRD	cysteine rich domain
CTF	C-terminal fragment
CuBD	copper-binding domain
dH_2O	destilled water
DMEM	Dulbecco`s Modified Eagle Serum
DMSO	dimethyl suloxide
DNA	desoxyribonucleic acid
DR6	death receptor 6
DTT	Dithiothreitol
E2	conserved region of the central APP domain
EGF	epidermal growth factor
ER	endoplasmic reticulum
EST	expressed sequence tags
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
GFLD	N-terminal growth factor-like domain
h	hour(s)
HaCaT	Human adult low Calcium high Temperature Keratinocytes
Hek	human embryonic kidney
I	inserted
Il	interleukin
ISH	in situ hybridization
iTRAQ	isobaric tags for relative and absolute quantitation
kDa	kilodalton
KLK	kallikrein
KPI	Kunitz protease inhibitor
Lac	lactose operon
mAb	monoclonal antibody
MAM	meprin A5 protein, receptor tyrosine phosphatase μ
MCS	multiple cloning site

meprin	metalloprotease from renal tissue
met	methionine
min	minute
mM	milli mole
MMP	matrix metalloprotease
mTld	mammalian tolloid
Ox2	Orexin receptor type 2
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC7	proprotein convertase 7
PICS	Proteomic Identification of protease Cleavage Sites
PMA	phorbol 12-myristate 13-acetate
PPH	PABA peptide hydrolases
pro	propeptide
PSEN	presenelin
PTM	posttranslational modification
PVDF	polyvinylidene fluoride
s	seconds
SDS	sodium dodecyl sulfate
Sw	Swedish
TACE	tumor necrosis factor-α converting enzyme
TAILS	Terminal Amine Isotopic Labeling of Substrates
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
TGF	TNF α-converting enzyme
TGN	trans-Golgi network
Tll-1, -2	tolloid like-1 and -2
TM	transmembrane
TNF	tumor necrosis factor
TRAF	TNF-receptor associated factors
TRIS	Tris-(Hydroxymethyl)aminomethan
U	unit
U373	glioblastoma-astrocytoma cells
V	volt
v	velocity
VEGF-A	vascular endothelial growth factor A
wt	wildtype

9.6 ONE AND THREE LETTER CODE OF AMINO ACIDS

one letter code	three letter code	amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

CURRICULUM VITAE

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DECLARATION OF AUTHORSHIP

I hereby confirm that I have authored this PhD thesis independently and without use of other than the indicated resources.

Mainz, November 11th 2011

(Tamara Jefferson)