ABSTRACT

Proposed Regulatory Role of Noncatalytic ADAMs in Ectodomain Shedding

by

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Members of the ADAM (A Disintegrin And Metalloprotease) protein family uniquely exhibit both proteolytic and adhesive properties. Specifically, ADAMs catalyze the conversion of cell-surface proteins to soluble, biologically active derivatives through a process known as ectodomain shedding. Ectodomain shedding coordinates normal physiological processes. Aberrant ADAM activity contributes to pathological states, such as chronic inflammation. Understanding how ADAM ectodomain shedding activity is governed may provide new avenues for therapeutic intervention of ADAM-mediated shedding pathologies.

While ectodomain shedding is the hallmark feature of the ADAMs, thirteen of the forty ADAMs identified among various species are catalytically inactive. Noncatalytic ADAMs lack one or more consensus elements (HExxHxxGxxH) within the active site of the metalloprotease domain. Despite lacking the hallmark catalytic activity, noncatalytic ADAMs exhibit function(s) associated with other nonenzymatic domains (e.g. integrin

recognition of the disintegrin domain). Disruption/mutation of noncatalytic ADAMs has been associated with perturbation of biological events.

My overall hypothesis is that noncatalytic ADAMs regulate the activity of catalytically active ADAMs by competing for substrates and/or receptors when expressed within the same cellular niche. To begin testing this proposed competitive binding regulatory mechanism, I used noncatalytic human ADAM7 and catalytically active human ADAM28 as a model ADAM pair. Preliminary, unpublished data from our lab demonstrated expression of ADAM7 mRNA in multiple immune cell lines established to express ADAM28 at the protein level. For determination of ADAM7 expression patterns, monoclonal antibodies against ADAM7 were produced by our lab. However, the antibodies failed to exhibit reactivity against exogenous, full-length ADAM7.

Based upon preliminary phylogenetic analysis and genomic location, it is likely that ADAM7 arose from gene duplication of ADAM28, which would allow a genetic copy of the molecular specificity required for regulation (e.g. integrin binding) with eventual silencing of catalytic activity. We predicted that the gross structural integrity of the metalloprotease may be uniquely conserved between ADAM7 and ADAM28. Restoration of the active site glutamate residue of the ADAM7 metalloprotease domain bestowed catalytic activity to ADAM7 in a manner that reflected specificity of ADAM28-mediated catalysis. This is the first demonstration, to our knowledge, of “awakening” a noncatalytic enzyme through a single point mutation. This discovery provides an initial functional link between ADAM7 and ADAM28 and lends credence to the hypothesis that ADAM7 may regulate ADAM28 through competitive binding. These findings have a broader impact, as 92 of the 570 collective human proteases are noncatalytic.

# Proposed Regulatory Role of Noncatalytic ADAMs in Ectodomain Shedding

A Thesis

Presented to the Faculty of the Department of Biochemistry and Molecular Biology

East Carolina University

In Partial Fulfillment of the Requirements for the Master's of Science Degree in Biomedical Sciences

by

Jason Andrew Hoggard

July 2015

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## LIST OF ABBREVIATIONS

EGTA – Ethylene Glycol Tetraacetic Acid

EDTA – Ethylenediaminetetraacetic Acid

PMSF – Phenylmethylsulfonyl Fluoride

GPCR – G-Protein Coupled Receptor

HB-EGF – Heparin-Binding Epidermal Growth Factor

IGFBP-3 – Insulin-like Growth Factor-Binding Protein 3

IGF-1 – Insulin Growth Factor 1

NHS – N-hydroxy Succinimide

PVDF – Polyvinylidene Difluoride Membrane

HRP – Horseradish Peroxidase

FITC – Fluorescein Isothiocyanate

DMEM – Dulbecco’s Modified Eagle’s Medium

PBST – Phosphate-Buffered Saline with Tween 20

## Introduction

ADAMs (A Disintegrin And Metalloprotease) are members of the Adamalysin subfamily of metzincin metalloproteinases (Gromis-Rüth, 1993), but they are unique compared to other proteases in that they also exhibit adhesive properties (Edwards, 2008). Currently, 40 total ADAMs have been identified across many species, ranging from yeast (*Schizosaccharomyces pombe)* and nematodes (*Caenorhabditis elegans)* to vertebrate animals such as *Xenopus laevis* and humans (Huxley-Jones, 2007). Irrespective of the species, each ADAM is expressed as a zymogen consisting of a prototypical multidomain architecture. Listed from N-terminus to C-terminus, the domains are: Pro-domain, Metalloprotease domain, Disintegrin domain, Cysteine-rich domain, EGF domain, transmembrane domain, and the Cytoplasmic domain (Fig 1) (Weber, 2012). The hallmark feature of the ADAM family is ectodomain shedding, which is the cleavage of cell surface substrates into soluble, biologically active derivatives (Fig 2).

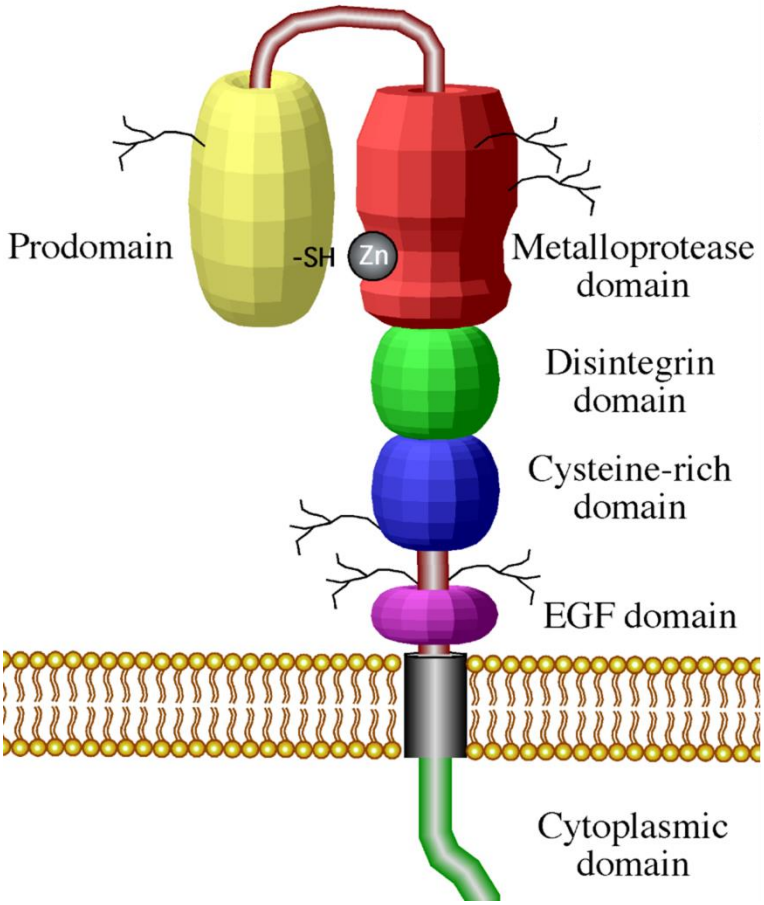
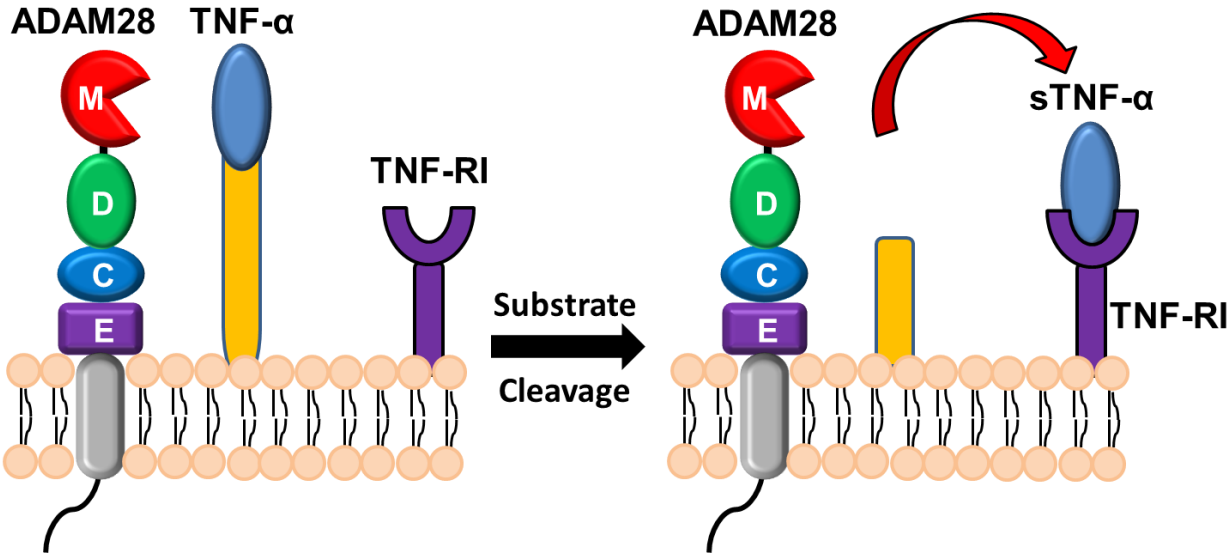

Prodomain
-SH
Zn
Metalloprotease domain
Disintegrin domain
Cysteine-rich domain
EGF domain
Cytoplasmic domain

**Figure 1. Prototypical ADAM Domain Architecture.** Depicted are the multiple, functional domains typical of the ADAM protein family. Multi-domain architecture is conserved across catalytically active and noncatalytic ADAMs. Soluble ADAM isoforms that arise from alternative splicing of selective members terminate following the EGF domain. Conserved N-glycosylation sites important for structure and function are displayed as branches extending from the ADAM structure.


ADAM28
TNF-α
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TNF-RI
Substrate
Cleavage
ADAM28
sTNF-α
M
D
C
E
TNF-RI

**Figure 2. ADAM-mediated Ectodomain Shedding.** Catalytically active ADAM28 processes membrane-bound TNF-α into soluble TNF-α (sTNF- α). sTNF- α is freely diffusible and able to bind TNF-Receptor I (TNF-RI) on the same cell surface or an adjacent cell. Binding of sTNF- α to TNF-RI stimulates signaling cascades to promote inflammation (Jowett, 2012).

## ADAM Domains

The N-terminal pro-domain is responsible for maintaining latency of catalytic activity through a cysteine switch mechanism (Loechel, 2002; Milla, 2006; Roghani, 1999; Smith and DeSimone, 2002). The cysteine switch prohibits zinc coordination within the catalytic active site to prevent catalysis. Latency is maintained by the cysteine switch through steric obstruction of the active site and coordination of $Zn^{2+}$ via a conserved cysteine residue within the pro-domain that positions the sulfur of its side-chain in close proximity of $Zn^{2+}$ within the active site (Loechel, 1999). A similar regulatory strategy is exhibited by Matrix Metalloproteases (MMPs), a family of metzincin proteases similar to the ADAMs (Bode, 1993). Removal of the pro-domain occurs in the trans-Golgi network during post-translational processing by self-activation of the metalloprotease domain (Lum, 1998; Schlomann, 2002) or by the action of proprotein convertases such as furin proteases (Lum, 1998). In addition to maintaining latency of the catalytic active site, the pro-domain acts as a chaperone post-translationally by aiding folding and trafficking of the ADAM prior to being removed (Roghani, 1999).

The metalloprotease domain is responsible for ectodomain shedding of substrates, which occurs via nucleophilic attack of the peptide backbone of peptide substrates (Fig 3). The active site within the metalloprotease domain contains a consensus sequence (HExxHxxGxxH) that is indicative of metal ion coordination and nucleophilic attack of the peptide substrates (Bode, 1993). The achiral nature of glycine allows for a turn within the structure of the active site, providing the necessary flexibility required for positioning the three histidine residues required for zinc coordination (Fig

4). Zinc plays a role in coordinating water molecules to the active site. The deprotonated carboxylic acid side chain of glutamate acts as a nucleophile, extracting a proton from water molecules coordinated to the $Zn^{2+}$ metal center. The deprotonated water molecule then acts as a nucleophile toward a carbonyl carbon of the peptide backbone of the target substrate (Gromis-Rüth, 2003). A conserved methionine turn within the catalytic domain, a characteristic feature of metzincin proteases, is highly conserved among the ADAMs and is thought to play a role in zinc coordination (Bode, 1993). X-ray crystallographic studies of SVMPs, VAP1 and VAP2B, have revealed much of what is known about the structure of the metalloprotease domain (Igarashi, 2007; Takeda, 2006). The metalloprotease domain consists of two distinct subdomains, with the catalytic active site positioned within a cleft created by the subdomains. The N-terminal subdomain is composed of four α-helices and a highly twisted five-stranded β-sheet. The lower subdomain is composed of a single α-helix and an unevenly folded portion, which is involved in recognition of substrates (Takeda, 2009).

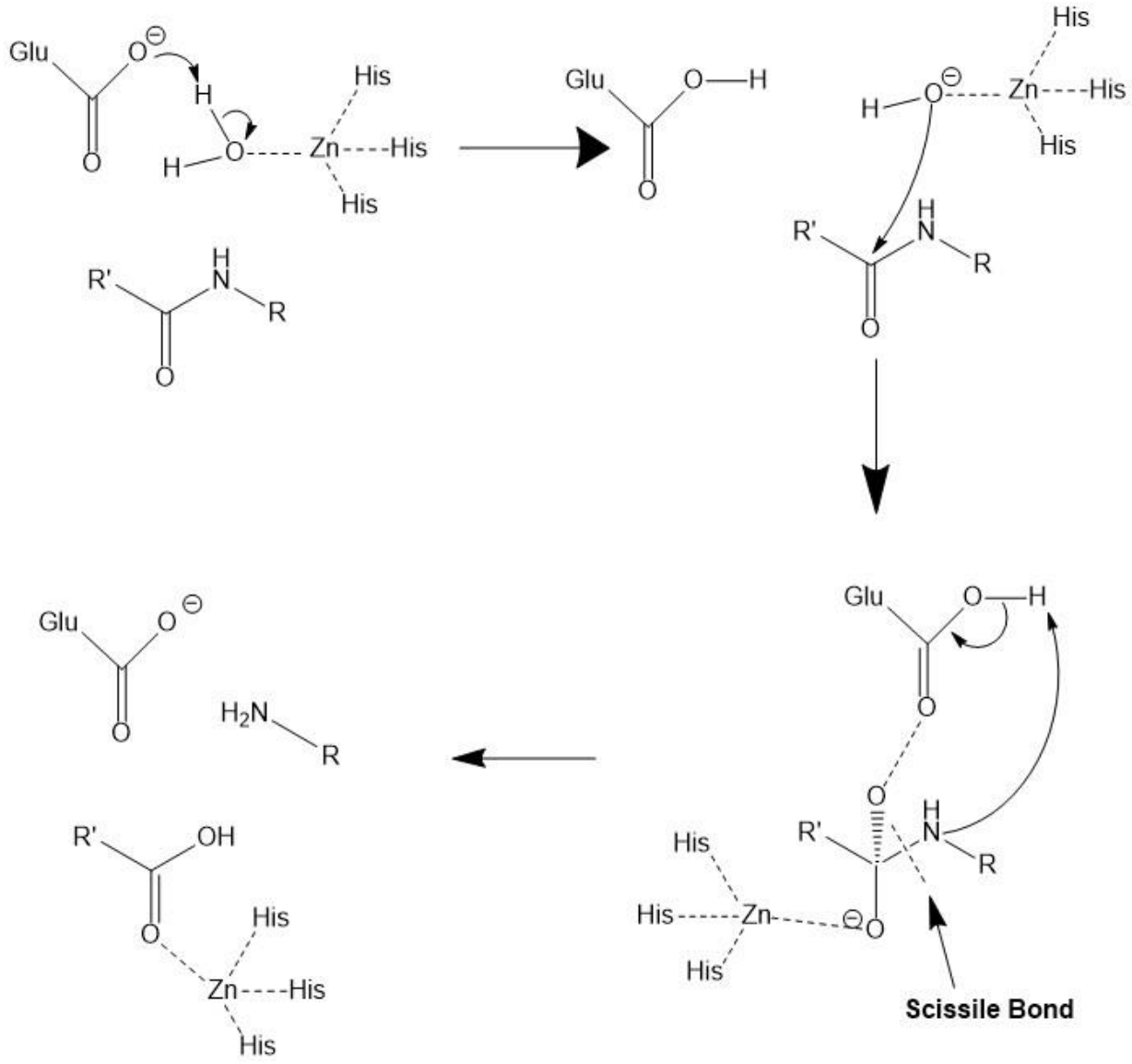
While metalloprotease function is one of the most defining and salient aspects of the ADAM family, nearly half of the human ADAMs lack one or more of the consensus sequence elements required for catalytic activity (Wei, 2011). However, these noncatalytic ADAMs still exhibit the overall domain architecture of a prototypical ADAM (Fig 1) (Liu, 2009). Biological implications of these noncatalytic ADAMs have begun to emerge in the literature as a result of investigating expression knockdown or protein mutations. Mutations in ADAM7 have been linked to increased metastasis of melanoma cells, which is speculated to be a result of decreased adhesion of extracellular matrix components, such as collagen IV and laminin-1 (Wei, 2011). Despite these studies, the

biological significance of these fairly abundant “dead” sheddases is still poorly understood.

The list of ADAM substrates is vast and encompasses molecules such as cytokines, growth factors, growth factor receptors, and GPCR ligands (Hartmann, 2013). ADAM-mediated shedding has been implicated in the coordination of many physiological processes, including embryonic development and immune response. One example is the shedding of neuregulin via ADAM19, which has been documented to play a crucial role in cardiac development (Zhou, 2004). Additionally, Tumor Necrosis Factor-α (TNF-α) shedding via ADAM28 (Jowett, 2012) results in a pro-inflammatory signaling cascade during immune response, excess shedding of which has been implicated in chronic inflammation. While these processes involve many additional signaling events and molecular interactions, ADAM-mediated shedding plays a vital role in many physiological events.

As ADAM proteolytic action is required for many critical events from development into adulthood, dysregulation of ADAM-mediated shedding can be deleterious. ADAM-mediated shedding of biologically decisive molecules, such as growth factors and cytokines, place ADAMs at the interface of health and disease (Klein, 2010; Weber, 2012). For example, while TNF-α shedding, cleavage of the TNF-α ectodomain into a biologically active form, plays a role in the normal health state, excess shedding of TNF-α by ADAM17 has been implicated in chronic inflammatory states, such as rheumatoid arthritis (Klein, 2010). Additionally, increased levels of soluble TNF-α can lead to increased transcription of ADAM17 (Bzowska, 2004), compounding and further promoting the inflammatory state in a vicious cycle. Excess shedding of heparin-

binding epidermal growth factor (HB-EGF) via ADAM12 has been implicated in cardiac hypertrophy (Asakura, 2002), a condition that can result in cardiac failure and eventually death. As these examples illustrate, dysregulation of ADAM-mediated shedding can play a role in pathological conditions, many of which lack efficient treatment. An enhanced understanding of how ADAM-mediated shedding is regulated is absolutely crucial to developing new avenues of treatment for a spectrum of disease states.


Glu
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O—H
R'
N
R
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His
Zn
His
Scissile Bond
Glu
O
$H_2N$
R
R'
OH
His
Zn
His
His

**Figure 3. Nucleophilic Attack Of a Peptide Substrate.** Zinc, coordinated by three histidine residues, coordinates water molecules to the metal center within the active site. Glutamate deprotonates water. The deprotonated oxygen of water acts as a nucleophile toward the carbonyl carbon of the peptide backbone of the substrate, resulting in cleavage at the scissile bond.

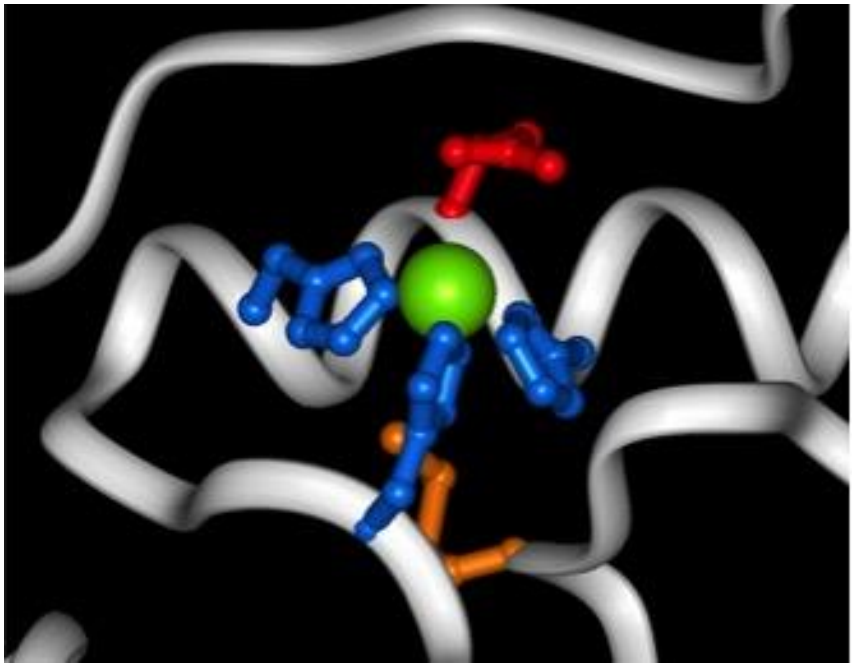


**Figure 4. ADAM Metalloprotease Domain Catalytic Active Site.** ADAM28 catalytic active site modeled on the catalytic active site of ADAM33. The catalytic active site in the metalloprotease domain possesses a consensus sequence (HExxHxxGxxH) indicative of catalytic activity. Glycine provides a structural turn, allowing the three histidine residues (represented in blue) to coordinate $Zn^{2+}$ (represented by the green sphere) within the active site. Glutamate (represented in red) is required for nucleophilic attack.

The disintegrin domain provides a means for integrin receptor recognition by ADAMs. The integrin ligand properties of ADAMs have been reported to facilitate cell-cell and cell-matrix interactions and promote cell migration (White, 2003). In fact, a significant amount of overlap exists among the ADAMs with respect to integrin recognition and interaction. For example, seven different ADAMs (ADAM2, ADAM3, ADAM7, ADAM12, ADAM19, ADAM28, and ADAM33) serve as integrin α4β1 ligands (Arribas, 2006). Despite the overlapping integrin ligand specificities, studies investigating whether ADAMs compete for integrin occupancy are lacking. Preliminary unpublished work from our lab is the first to demonstrate that the ADAM disintegrin domains selectively compete for integrin receptor binding (Fig 5).

The concept that ADAMs compete for integrin receptors is novel and forms the basis for our regulatory model (Fig 6). By exhibiting competition for integrin receptor occupancy, it is highly plausible these ADAMs compete for additional substrates using their nonenzymatic domains. In fact, competitive binding of substrates in this manner may govern the availability of substrates, which is the basis of the proposed regulatory role.

In addition, emerging evidence in the literature indicates that the nonenzymatic domains (disintegrin and cysteine-rich domains) of the ADAM family confer specificity to the metalloprotease domain (Smith and DeSimone, 2002; Xu, 2010; Sadler E., 2009; Stawikoska, R., 2013) and that integrin recognition is critical in governing ADAM17-mediated shedding (Gooz, 2012). Given these studies demonstrating the importance of the disintegrin and cysteine-rich domains to ADAM-mediated shedding, the presence of catalytically inactive ADAMs that exhibit adhesive properties indicates the potential for

similar interactions that may play a role in regulating substrate availability. However, no explorations into how these formative findings relate or apply to the appreciable number of noncatalytic ADAMs have been reported.

In addition to the adhesive properties of the disintegrin domain, the cysteine-rich domain aids adhesion to extracellular matrix (ECM) components. In fact, this function is independent of disintegrin-integrin interactions (Klein, 2010). A key, defining feature of the cysteine-rich domain is the hypervariable region (HVR), which is speculated to play a role in recognition of substrates important to cell-cell and cell-matrix interactions (Takeda, 2006; Igarashi, 2007). This has led to speculation that the cysteine-rich domain works concomitantly with the disintegrin domain to confer specificity to the metalloprotease domain (Takeda, 2006).

The EGF domain is the C-terminal end of the ectodomain. While the EGF domain function has not been determined, it is thought to act as a spacer to maintain distance from the cell surface and other extracellular domains to facilitate interactions with substrates and receptors. Following the EGF domain is the transmembrane domain, anchoring the protein to the cell surface. Transcription of soluble ADAM isoforms (e.g. ADAM8, ADAM9, ADAM12, ADAM28, and ADAM33) is terminated following the EGF domain via alternative splicing (Klein, 2011).

The cytoplasmic domain is the only intracellular domain and is the site of the most variation in length and protein sequence among the ADAMs (Seals and Courtneidge, 2003; Takeda, 2006). The cytoplasmic domain contains putative phosphorylation sites for serine-threonine and tyrosine kinases, as well as providing

potential binding sites for SH3 and SH2 domain-containing proteins (Howard, 1999). Interestingly, intracellular interactions involving the cytoplasmic domain have been shown to play a role in regulating the specificity of shedding activity exhibited by the metalloprotease domain (Xu, 2010). The work by Xu *et al.* provides additional evidence that nonenzymatic domains and their respective molecular interactions serve to modify activity of the metalloprotease domain, and therefore, embody a potential regulatory mechanism. Due to the diversity exhibited within the cytoplasmic regions of ADAMs, we do not posit that noncatalytic ADAMs regulate shedding by competing for cytosolic binding partners. However, the possibility of competition for cytosolic binding partners will be addressed by the Bridges lab in future cell-based experiments. If noncatalytic ADAMs do govern shedding activity, inside-out signaling, in which an intracellular signaling event produces an extracellular effect, involving their cytoplasmic domain could potentially play a role in their ability to interact with substrates via the metalloprotease domain.

**Regulation of ADAM-Mediated Shedding**

Established mechanisms for regulating shedding include the modulation of ADAM transcription, the removal of the pro-domain, trafficking of ADAMs within the cell and escort to secretory pathways, and removal of ADAMs from the cell surface via endocytosis (Reviewed by Hartmann, 2013) (Table 1). Additionally, phosphorylation of the cytoplasmic tail has been shown to activate shedding via inside-out signaling (Xu, 2010). Binding of substrate with the disintegrin or cysteine-rich domain can also play a regulatory role. For example, the cysteine-rich domain is required for shedding of IL-1 Receptor-II by ADAM17 (Reddy, 2000). Interaction of the disintegrin domain with

integrins comprises an alternate mechanism of regulation. An example of integrin-ADAM interaction affecting shedding is the subsequent decrease and inhibition of shedding activity by ADAM17 (Gooz, 2012).

However, regulation is not limited to the enzyme level. Only a small, select group of catalytically active ADAMs is responsible for shedding a large number of diverse substrates (Huovila, 2005). Therefore, it seems reasonable that regulation also occurs at the level of substrate (Hartmann, 2013). Regulatory mechanisms may include post-translational modification and conformational change. Another example of substrate modification impacting catalytic activity is the binding of Notch receptor with ligand, which is required for ADAM10-mediated shedding of the Notch1 receptor ectodomain (Bozkulak, 2009).

The regulatory mechanisms emerging in the literature provide a relevant context in which to finally address the biological role of noncatalytic ADAMs. Specifically, the nonenzymatic domains (disintegrin and cysteine-rich domains) of a catalytically active ADAM demonstrating a role in specificity and co-localization to facilitate shedding provides a glimpse into the potential function of noncatalytic ADAMs. We believe the nonenzymatic domains of noncatalytic ADAMs may serve a similar role through interactions with substrates typically shed by their catalytically active counterparts.

**Table 1.** *Regulatory Mechanisms of Shedding*

| Mechanism | Identified Cellular Consequence | Reference |
|---|---|---|
| Removal of pro-domain | Activation of metalloprotease domain | Roghani, 1999; Lum, 1998; |
| Modulation of transcription | Apoptosis, cell proliferation, cancer progression | Mochizuki, 2007 |
| Removal from cell surface | Decreased shedding, altered signaling, disrupted substrate function | Carey, 2011 |
| Phosphorylation of cytoplasmic tail | Cell proliferation, cancer progression, and chronic inflammation | Xu, 2010 |
| Substrate conformational change | Notch1 conformational change exposing cleavage site and facilitating selective cleavage to activate transcription | Bozkulak, 2009 |
| Unknown | Noncatalytic ADAMs govern substrate availability via competitive binding | Hypothetical Model for Current Thesis Proposal |

## Noncatalytic ADAMs

As with the ADAM field at large, the introduction has predominantly focused on the catalytic action of ADAM sheddases. However, 8 of the 21 human ADAMs are noncatalytic (Edwards, 2008) (Table 2). These ADAMs are defined by lacking one or more consensus elements (HExxHxxGxxH) within the catalytic active site. Despite missing these elements, they still possess the prototypical multi-domain architecture of their catalytically active counterparts (Liu, 2009). In spite of their prevalence, the biological relevance of ADAMs that lack hallmark sheddase activity is not well understood.

While the metalloprotease domain of the noncatalytic ADAMs does not exhibit catalytic activity, the noncatalytic domains exhibit function (e.g. integrin recognition) and select noncatalytic ADAMs have newly identified roles in biological processes. For example, ADAM23, a noncatalytic ADAM, has been implicated in axon guidance and neural connectivity during normal embryonic development (Leighton, 2001), as well as modulating activation of integrin αvβ3 (Verbisk, 2009) through its noncatalytic domains. ADM-1, a noncatalytic ADAM expressed in *C. elegans*, has also been implicated in neuron axon guidance, a function attributed to its nonenzymatic domains (Huang, 2003). Disruption of noncatalytic ADAMs has also been associated with pathological states. Mutations in the ADAM7 gene have been implicated in increased cell migration and metastasis of melanoma cells as a result of the decreased ability of ADAM7 to bind laminin-1 and collagen IV (Wei, 2011).

Although these studies attest to the biological relevance of noncatalytic ADAMs, they comprise only a few examples of their functional relevance. Based upon the frequency of noncatalytic ADAMs across all species (33%), one might expect an equivalent amount of articles would describe studies involving a noncatalytic ADAM. However, only 69 out of 1,039 (6.6%) articles retrieved via PubMed search of primary articles containing ADAM nomenclature in the title investigated noncatalytic ADAMs.

Because they are severely understudied, it is likely that noncatalytic ADAMs are exerting biological effects that have yet to be delineated. What we believe, as is expanded on in my hypothesis, is that noncatalytic ADAMs may play a role in regulating the activity of their catalytically active counterparts by governing access to or availability of substrates. This novel functional paradigm is supported by the fact that: *i)* a particular ADAM's noncatalytic domains contribute to catalytic activity and/or specificity of that specific ADAM, *ii)* both catalytically and non-catalytically active ADAMs contain the prototypical domains, possibly allowing for the same molecular interactions to occur, and *iii)* our preliminary data suggest that there is select competition between catalytic and noncatalytic ADAMs for receptor occupancy (Fig 5).

**Table 2.** *Noncatalytic ADAMs Expression Patterns and Roles in Health and Disease*

| Gene | Expression | Normal Health Roles | Pathological Implications* | Consensus Sequence** |
|---|---|---|---|---|
| ADAM2 | Testis | Sperm-egg interaction | Lung Carcinoma | QLLSLSMGITYD |
| ADAM7 | Testis, Erythrocytes | Cell-cell and cell-matrix interaction | Hepatic Carcinoma, Melanoma metastasis | HQLGHNLGMQHD |
| ADAM11 | Erythrocytes, Central & Peripheral Nervous Systems, Liver | Spatial learning, Motor coordination | Impaired spatial learning, Altered nociception | QTLGQNLGMMWN |
| ADAM18 | Testis, Erythrocytes, Bone marrow, Pancreas | Spermatogenesis, Fertilization | CNS Glioma, Malignant Melanoma | QLLGLNVGLTYD |
| ADAM22 | Central & Peripheral Nervous System | Mediates growth inhibition | Epilepsy, Peripheral neuropathy | |
| ADAM23 | Central & Peripheral Nervous System, Heart | Axon guidance, neural connectivity | Promotion of metastasis | QSLAQNLGIQWE |
| ADAM29 | Testis | Spermatogenesis, Fertilization | Malignant Melanoma, CNS Glioma | HHLGHNLGMNHD |
| ADAM32 | Testis, Blood lymphoid cells | Sperm-egg interaction | Hepatic Carcinoma | QMLALSLGISYD |

* Resulting from aberrant expression and/or mutations

**Consensus sequence of catalytically active ADAMs: **HExxHxxGxxH**

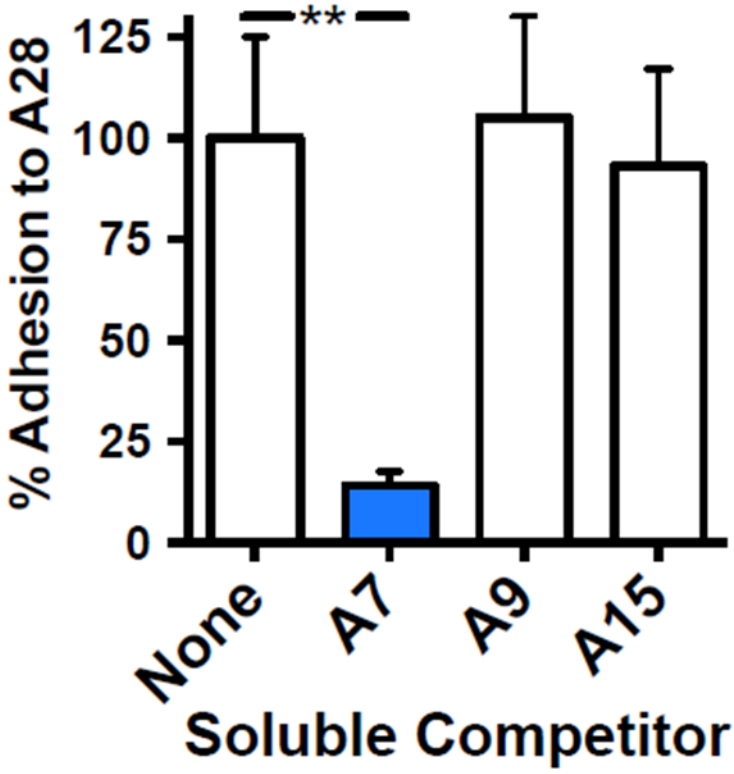

**
125
100
75
50
25
0
% Adhesion to A28
None
A7
A9
A15
Soluble Competitor

**Figure 5. Competitive Binding of ADAM Disintegrin Domains.** Soluble disintegrin domains of ADAM7, ADAM9 and ADAM15 were utilized in the presence of immobilized ADAM28 to investigate competitive binding of integrin receptors. ADAM7 alone competitively inhibits integrin-mediated adhesion to ADAM28 (*blue bar*). ADAM9 and ADAM15 did not result in a statistically significant difference in integrin-mediated cell adhesion to ADAM28 (white bars). Asterisks denote statistical significant ($p$<0.01) using one-way ANOVA with a Tukey's post-*hoc* test. Error bars represent standard error from the mean. Preliminary data obtained by the Bridges lab.

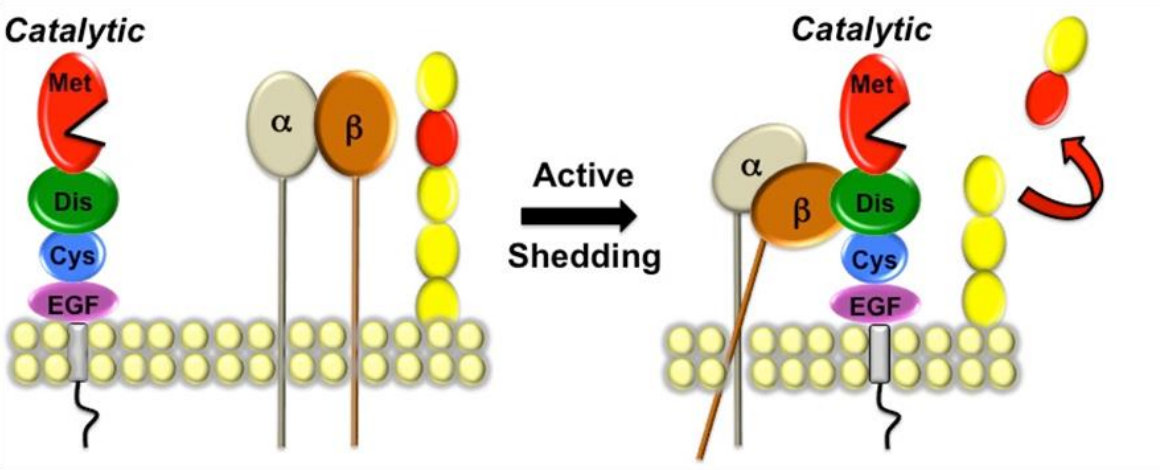
**Hypothesis**

As it is becoming apparent that nonenzymatic domains of catalytically active ADAMs contribute to regulation of shedding, noncatalytic ADAMs may potentially function in a similar manner. As the noncatalytic ADAMs can facilitate similar molecular interactions as catalytically active members, ***our lab's long-term hypothesis is that noncatalytic ADAMs govern ADAM-mediated shedding by competitively binding substrates and/or receptors*** (Fig 6). To begin determining whether this proves to be true, I have focused on investigating the structural preservation of the catalytic active site within the metalloprotease domain of noncatalytic ADAMs. My hypothesis, specific for my thesis studies, is that restoration of consensus elements within the active site of the metalloprotease domain via a single point-mutation will bestow catalytic activity, and specificity, to ADAM7 as is exhibited by its catalytically active counterpart, ADAM28. The "awakening" of catalytic activity would indicate the gross structural preservation of the catalytic active site in noncatalytic ADAMs.

Based upon this model, I posit that noncatalytic ADAMs arose from catalytic ADAM ancestors through gene duplication events. This would allow a genetic copy of the molecular specificity required for regulation (e.g. integrin binding) with eventual silencing of the catalytic activity by elimination of consensus site elements. The current work addresses the validity of the novel regulatory model by determining: i) catalytic and noncatalytic ADAMs exhibit an overlapping expression pattern to allow for competition and ii) that noncatalytic ADAMs possess remnants of catalytic activity. This study seeks to determine the preservation of the catalytic active site of noncatalytic ADAMs, as well as expression patterns of noncatalytic and catalytically active ADAM counterparts within

the same cellular niche. Results from my study are aimed at defining a novel function of noncatalytic ADAMs by providing the first evidence that noncatalytic ADAMs serve as regulators of ADAM-mediated shedding.

## A) Catalytic ADAMs without Competition




## B) Noncatalytic ADAMs Compete for Substrates

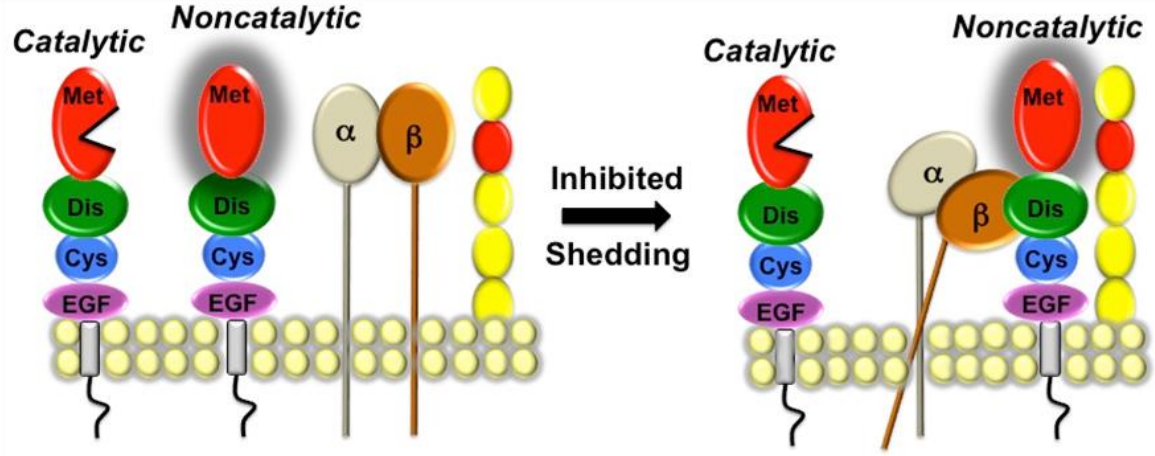


**Figure 6. Proposed Regulatory Role for Noncatalytic ADAMs.** A) Catalytically active ADAMs are free to bind receptors and shed substrates in the absence of a noncatalytic competitor. B) A noncatalytic ADAM binds an integrin receptor preventing the localization of a catalytically active ADAM to substrate. In contrast to panel A, competition between ADAMs prevents shedding.

**Approach and Model System (ADAM28 and ADAM7)**

To investigate my hypotheses, I used ADAM28 and ADAM7 as a model pair. Genes for both reside within a single cluster on human chromosome 8p21.2 (Bates, 2002), indicating ADAM7 may have arisen from gene duplication events of ADAM28. ADAM28 has previously been shown to be expressed at the protein level on the surface of many immune cell lines, including THP-1 monocytes and various B-lymphomas (McGinn, 2011). ADAM28, expressed as a 115kDa zymogen, is self-activated (Howard, 2000) and exists as an 88kDa protease in its mature form on the cell surface. In addition to expression on cell surfaces, ADAM28 is expressed as a soluble isoform due to alternative splicing. ADAM28 has been implicated in many physiological processes, including aiding transmigration of lymphocytes via integrin interactions (McGinn, 2011). Pathologically, excess ADAM28-mediated TNF-α shedding plays a role in chronic inflammation (Jowett, 2012), in states such as rheumatoid arthritis, and also has been implicated in osteoarthritis via the degradation of proteoglycans when expressed on the surface of chondrocytes (Hikichi, 2009). Additionally, ADAM28 has also been implicated in cell proliferative processes through the shedding of insulin-like growth factor binding protein-3 (IGFBP-3) of the IGFBP-3/IGF-1 complex (Mochizuki, 2004; Mitsui, 2006; Ohtsuka, 2006).

While much is known about ADAM28, very little is known about ADAM7. Previously, ADAM7 was found in human seminal fluid and speculated to be secreted by the epididymis (Sun, 2000). At the mRNA level, ADAM7 has been shown to be expressed and mutated in melanoma cells, in which cell adhesion is reduced aiding metastasis (Wei, 2011). In addition to its suggested role in melanoma cells, ADAM7

has been widely considered as a fertility-centric protein based on murine studies with a potential role in the docking of egg with sperm via integrin-disintegrin interactions on the cell surface (Han, 2010).

Preliminary data obtained in our lab, by JianMing Chen, has established that ADAM7 mRNA is expressed in many of the same immune cell lines previously demonstrated to express ADAM28 (Fig 7). While the presence of mRNA does not always result in protein expression, the preliminary data is suggestive of a potential overlap in expression that could allow competition of ADAM7 and ADAM28 within those cells and niches. The protein sequence of ADAM28 and ADAM7 displays a very high degree of homology within the disintegrin domain (67% identical, 76% similar), and these two ADAMs interact with the same integrin receptors (Bridges, 2005). Importantly, ADAM7 selectively inhibits integrin-dependent cell adhesion to ADAM28 (Fig 5). Analysis of the consensus sequence within the metalloprotease active site shows ADAM7 differs by only a single residue, as it possesses a glutamine instead of the consensus glutamate (Fig 8). Given these similarities, I believe ADAM28 and ADAM7 are the ideal model pair for investigation of this proposed regulatory mechanism.

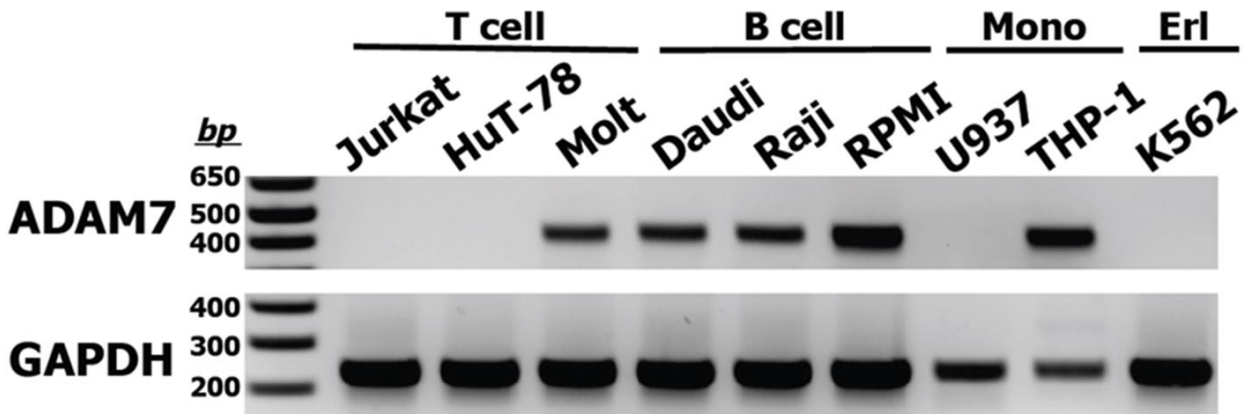

T cell
B cell
Mono
Erl
Jurkat
HuT-78
Molt
Daudi
Raji
RPMI
U937
THP-1
K562
bp
650
500
400
ADAM7
400
300
200
GAPDH

**Figure 7. Preliminary ADAM7 mRNA Expression Patterns.** Immune cell lines were investigated for ADAM7 mRNA expression via Reverse Transcriptase (RT)-PCR. Intron-spanning primers were utilized to discriminate products derived from genomic contamination. All products corresponded to amplification of cDNA templates and bands were sequence-verified to confirm the identity of ADAM7. Data obtained by JianMing Chen.

## Alignment of ADAM7 and ADAM28 Active Sites

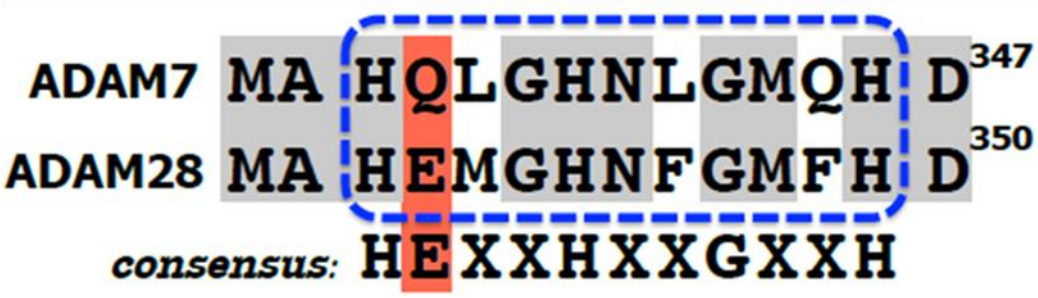


**Figure 8. ADAM7 and ADAM28 Active Site Alignment.** Alignment of the human ADAM28 and ADAM7 active site sequence. The consensus sequence is highlighted by the blue box. Shaded grey residues are conserved between ADAM7 and ADAM28. ADAM7 possesses all but one consensus element (highlighted in red).

## Experimental Procedures

### Reagents

Lipofectamine LTX and Lipofectamine PLUS transfection reagents were purchased from Invitrogen. Blasticidin-S was obtained from Corning. Anti-Fc HRP antibody from Sigma Aldrich was used for detection of Fc-fusion protein. Custom primers were ordered from Integrated DNA Technologies. EZ-Link Sulfo-NHS Biotin was purchased from Thermo Scientific. Catalytic activity and specificity assays used α2-Macroglobulin and myelin basic protein obtained from Sigma Life Sciences. 9e10 antibody was kindly provided by Dr. Judith White. Protein G Agarose Fast Flow resin and Immobilon PVDF membrane were obtained from Millipore. Goat anti-mouse Human adsorbed FITC secondary antibody was purchased from Southern BioTech.

### Cell Culture

High Five Insect cells were cultured in HyClone SFX-Insect Media (Fisher Scientific) supplemented with 1% penicillin-streptamycin and 0.1% gentamycin in a 27°C environment. CHO (Chinese hamster ovary) cell line was maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 1% penicillin-streptamycin, and 1% sodium pyruvate. HEK293 (human embryonic kidney) cell line was maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptamycin, and 1% sodium pyruvate. CHO and HEK293 cell lines were maintained at 37°C in a 5% $CO_2$ environment.

**Cell-Surface Biotinylation**

HEK293 and CHO cells were transfected with a full-length ADAM7 construct in pCS2+ vector or empty pCS2+ vector, termed 'mock', for 48 hours. Transfection of all cell lines was achieved with Lipofectamine LTX according to the manufacturer's recommendations. Following transfection, cells were washed twice in PBS (0.137M NaCl, 2.7mM KCl, 10mM $Na_2HPO_4$, 2mM $KH_2PO_4$), then incubated for 20 minutes in biotinylation buffer (PBS + 0.5µg/ml EZ-link NHS Biotin). The cells were washed twice in PBS and detached with 5mM EDTA in PBS. Detached cells were isolated via centrifugation at 1,000 rpm for 10 minutes at 4°C. The cell pellet was resuspended and incubated in lysis buffer (PBS, 1% Triton-100 and 1:100 Roche protease inhibitor cocktail) with rocking for 1 hour at 4°C. Cell surface proteins were isolated via centrifugation at 14,800 rpm for 10 minutes. Supernatant was subsequently incubated with avidin agarose beads while rocking at 4°C overnight. Beads were pelleted, supernatant decanted, and resin boiled in reduced SDS-PAGE sample buffer (500mM Tris-HCl pH 6.8, 10% SDS, glycerol, 2-β-mercaptoethanol, and 0.05% (w/v) bromophenol blue) prior to running on 10%/4% SDS-PAGE gel. Separated samples were transferred to a PVDF membrane via horizontal current. PVDF membrane was blocked for 1 hour at room temperature in 5% blotto (5% dry milk in PBST) with subsequent overnight incubation with the appropriate antibody (9e10 or ADAM7 mAb) at 4°C with agitation. Following overnight incubation at 4°C, the PVDF was prepared for development utilizing Advansta WesternBright ECL Kit.

## Flow Cytometry

HEK293 and CHO cells were transfected with a full-length ADAM7 construct in pCS2+ vector or empty pCS2+ vector for 48 hours and resuspended in FACS Buffer (PBS and 1% BSA) at $1x10^6$ cells/ml. Cells were incubated in the presence of ADAM7 monoclonal antibodies for 30 minutes at room temperature. Following application of the primary antibody, the cells were fixed in 0.37% formalin/PBS for 15 minutes. The cells were then washed twice in FACS buffer and stained with goat anti-mouse human-adsorbed IgG-FITC antibody for 15 minutes. For analysis of fluorescence intensity, cells were resuspended in 300µl FACS buffer and run via FACScan flow cytometer. Results were analyzed using Cell Quest-Pro software.

## Production of Recombinant DNA Constructs Encoding ADAM ProMet-Fc

DNA constructs were generated by extension of overlapping regions encoding the full pro-domain and metalloprotease domain of ADAM28 ($Iso^{19}$ – $Asp^{403}$) and ADAM7($Lys^{19}$ – $His^{400}$) to a 5' GP67 insect secretion signal and 3' human IgG3 Fc affinity tag. Following construct generation, PCR products were cloned into pIB/V5-His TOPO TA vector (Life Technologies) and sequence verified.

## QuickChange Mutagenesis of Human ADAM7 Pro-Met Construct

Restoration of $Glu^{337}$ within the catalytic active site was accomplished using QuikChange Mutagenesis PCR kit (Agilent Technologies). Primers were used to introduce a single, point mutation resulting in the substitution of cytosine to guanine at the first position within the codon for $Glutamine^{337}$ (5'-

CAGAATGGCACAT**G**AACTGGGGCATAAC-3' and reverse primer 5'-GTTATGCCCCAGTT**C**ATGTGCCATTCTG-3').

**Expression and Purification of Recombinant FC-fusion Protein**

Following sequence verification, High Five insect cells were transfected with 8μg of DNA constructs (ADAM28 PM-Fc wt, ADAM7 PM-Fc wt, and ADAM7 PM-Fc $Q^{337}$/E) using Lipofectamine PLUS transfection reagent in a 100mm dish at 75% confluency. 72 hours post-transfection, cells were selected for successful transfection using 80 μg/ml Blasticidin for 24 hours. Blasticidin concentration was then lowered to 50 μg/ml for continued maintenance and protein production thereafter. Protein-enriched medium was harvested and concentrated overnight with polyethylene glycol in dialysis tubing in PBS + 5mM EDTA (pH 7.4) at 4°C. Concentrated medium was applied to a protein-G affinity resin column, washed, and eluted via 100mM citric acid (pH 3.0) as 1ml fractions into tubes containing 300μl 1M Tris (pH 9.0) and 5mM EDTA. Eluted fractions were analyzed for the presence of protein, measuring absorbance at 280nm; protein-containing fractions were pooled and dialyzed overnight in PBS + 1mM EDTA (pH 7.0). Dialyzed fractions were then further concentrated via Amicon 30K MWCO filters (Millipore) according to the manufacturer's instructions. Concentrated eluent was quantified via BCA assay to determine final protein concentration.

**α2-Macroglobulin Trapping Assays**

Using previously established assay conditions (Loechel, 1998), trapping assays utilizing α2-Macroglobulin (α2M) were used to investigate catalytic activity. Individual reactions were set up with 0.5μg recombinant ADAM protein (ADAM28 PM-Fc wt,

ADAM7 PM-Fc wt, ADAM7 PM-Fc $Q^{337}$/E) and 30μg human plasma α2-Macroglobulin. α2M buffer (100mM NaCl, 50mM Tris-HCl (pH 7.4), 10mM $CaCl_2$, 0.02% Sodium Azide) was added to a final volume of 25μl. Reactions were incubated at 37°C for 24 hours, prior to being denatured and reduced and run on 7.5%/4% SDS-PAGE gel at 150V for 60 minutes. Resolved bands were transferred to PVDF membrane, blocked in 5% blotto (5% dry milk in PBST) for 1 hour at room temperature, and incubated overnight in the presence of anti-Fc antibody at 4°C with agitation. After extensive washing, the PVDF was prepared for development using Advansta WesternBright ECL Kit. To determine the relative contribution of zinc or glutamate, the assay was repeated with indicated concentrations of these parameters (Fig 11B and 11C).

**Myelin Basic Protein Catalytic Specificity Assay**

Assays were adapted and optimized from established techniques (Howard, 2001). To establish catalytic specificity, 25μl reactions were prepared using 1.5μg soluble, recombinant ADAM protein (ADAM28 PM-Fc wt, ADAM7 PM-Fc wt, ADAM7 PM-Fc $Q^{337}$/E), consisting solely of the pro-domain and metalloprotease domain with a C-terminal Fc-fusion tag, in the presence of 6μg myelin basic protein in MBP Assay Buffer (0.1M Glycine, 0.1mM Tris-HCl, 10mM $CaCl_2$, 0.5mM $ZnCl_2$ (pH 7.4)). Reactions were incubated at 37°C for 20 hours. Following incubation, reactions were denatured and reduced prior to being run on 15%/4% SDS-PAGE gel at 150V for 90 minutes. Upon completion of electrophoresis, the gel was washed 3x5 minutes in ultra-pure $H_2O$ and stained overnight in Imperial Stain (Thermo Scientific), after which the gel was destained in ultra-pure $H_2O$ for 5 hours.

## Results

### ADAM7 Monoclonal Antibodies Selectively React with Soluble ADAM7 Disintegrin Domain

A key component of the proposed regulatory mechanism is the expression of noncatalytic ADAMs in the same niche as their catalytically active counterparts. Our preliminary data demonstrated that ADAM7 mRNA is expressed in a manner that reflects patterns previously established for ADAM28 (Fig 7). Unfortunately, ADAM7 monoclonal antibodies are not commercially available or readily accessible to address the protein expression pattern of human ADAM7. Therefore, our lab had hybridomas developed with the goal of detecting full-length ADAM7 on the cell surface of human primary B-cells, immortal lymphoma lines, and tumor samples previously shown to express ADAM28.

Using a soluble ADAM7 disintegrin domain Fc-fusion protein (Dis-Fc) as the antigen, ten hybridoma lines were produced for our lab. After testing reactivity of the newly developed ADAM7 monoclonal antibodies in ELISA, supernatant from the ten hybridoma lines was tested for reactivity toward soluble ADAM7 disintegrin domain using Western Blot (Fig 9). To verify specificity toward ADAM7, each of the hybridoma lines were concurrently tested against soluble ADAM28 Dis-Fc protein. Several of the hybridoma lines (PD-2, 4, 5, 6, 7, 9, and 10) exhibited the desired ADAM7-selectivity and were able to recognize denatured and reduced ADAM7 Dis-Fc protein. As our ultimate goal is to identify the expression pattern of ADAM7 in various human immune

cells and cell lines through flow cytometry, I wanted to determine if the monoclonal antibodies were also able to recognize full-length ADAM7 natively expressed by cells.

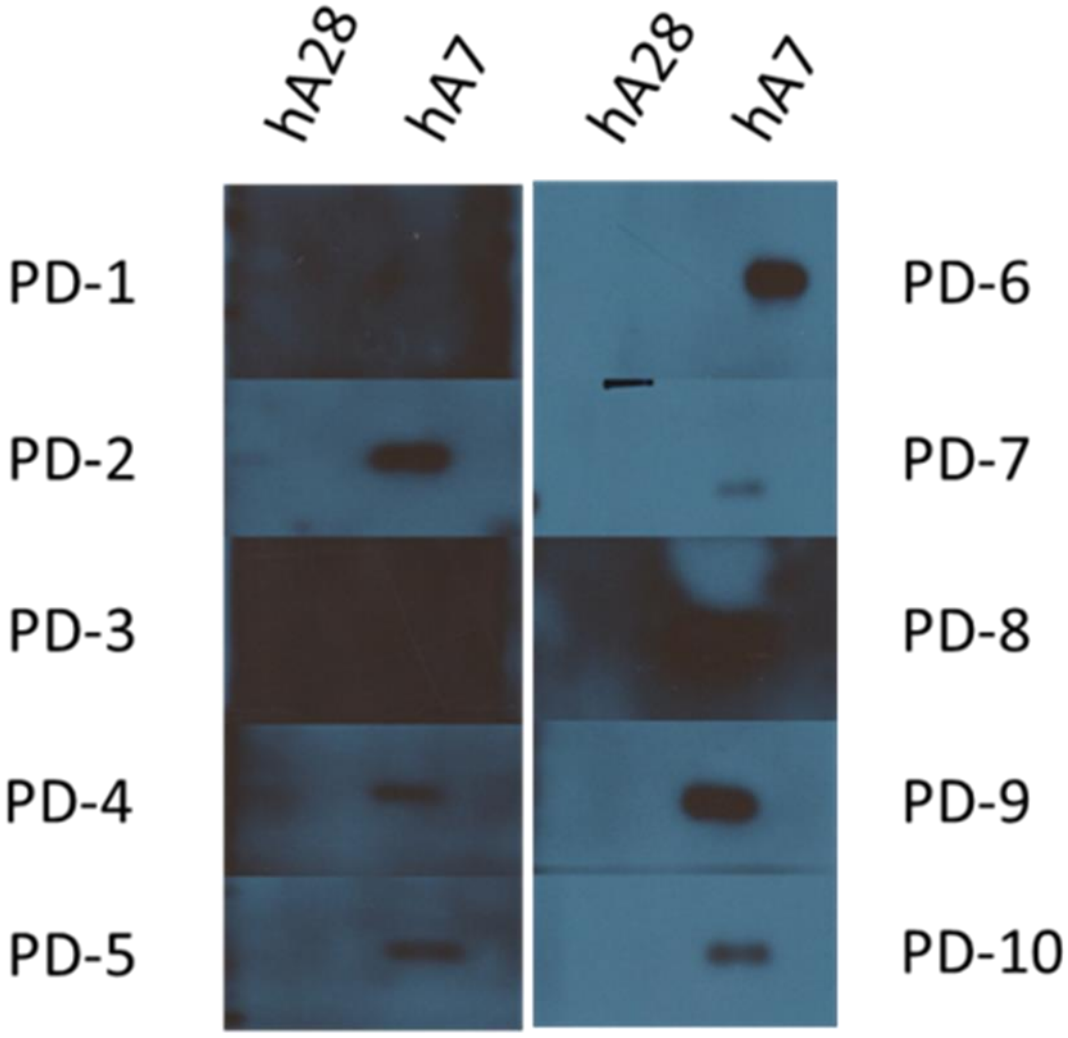

hA28
hA7
hA28
hA7
PD-1
PD-2
PD-3
PD-4
PD-5
PD-6
PD-7
PD-8
PD-9
PD-10

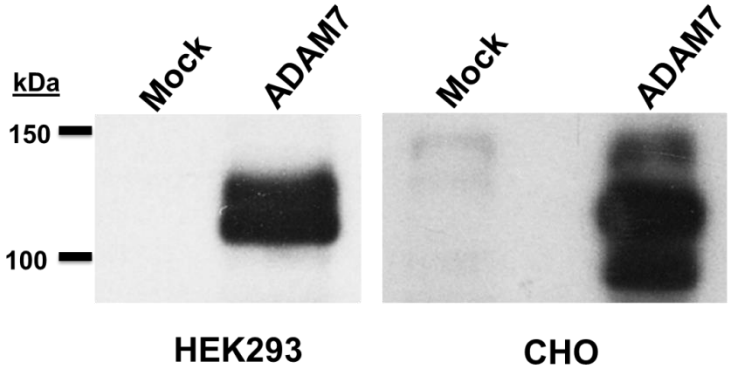
**Figure 9. Selective Reactivity of ADAM7 Monoclonal Antibodies.** Soluble ADAM28 and ADAM7 Disintegrin domain (3 µg/lane) was utilized to verify reactivity and selectivity of ADAM7 monoclonal antibodies.

**Recognition of Full-length ADAM7 Protein**

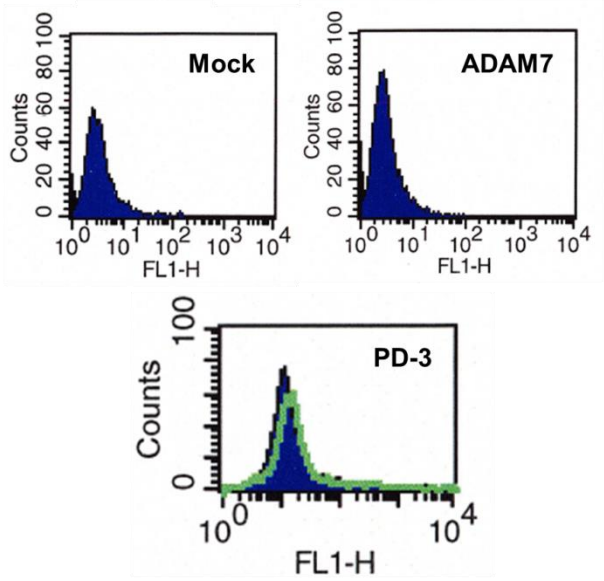
To assess if the antibodies could recognize full-length ADAM7 within a cell, I exogenously expressed human ADAM7 in HEK293 (human embryonic kidney) and CHO (Chinese hamster ovary) cell lines. The full-length ADAM7 construct, cloned into pCS2+ vector, contained a C-terminal Myc tag for detection purposes. Mock treatment consists of an empty pCS2+ vector. As shown in Figure 10A, exogenous cell-surface ADAM7 was observed in transfected cells, but not in the mock treated cells.

Initially, mock and ADAM7 transfected cells were analyzed for ADAM7 expression via flow cytometry with various dilutions of ascites from the monoclonal hybridomas. While a variety of conditions were tested, none produced shifts in fluorescent intensity correlating with ADAM7 expression (Fig 10B). Although the antibodies failed to work in flow cytometry, we speculated that the epitope may only be accessible when the protein is denatured. To test this possibility, cell surface proteins were selectively enriched from mock and transfected cells and analyzed under reducing conditions in an immunoblot. Unfortunately, no distinct bands corresponding to ADAM7 were observed when the cell surface proteins were immunoblotted (Fig 10C). Despite having multiple cell lines successfully express exogenous ADAM7, the hybridoma supernatant did not exhibit the ability to discriminate between mock and ADAM7-transfected cells in flow cytometry or immunoblot. Ability of the ADAM7 monoclonal antibodies to react with purified recombinant full-length ADAM7 was not assessed.

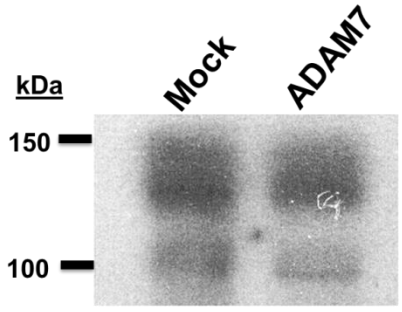
**A) ADAM7 Transfection Expression in Multiple Cell Lines**




**B) Flow Cytometry Detection of ADAM7**




**C) Cell-Surface Biotinylation Detection of ADAM7**




**PD-10**

**Figure 10. ADAM7 Monoclonal Antibody Detection of Full-Length ADAM7 on the Cell Surface.** A) The indicated cell lines were transfected with full-length human ADAM7 in the pCS2+ myc tag vector. Isolation of cell surface proteins was performed with biotinylation and SA-agarose pull down. Detection of myc-tag epitope with the mAb 9E10 was done to verify exogenous ADAM7 (~100 kDa). B) Flow cytometry analysis of mock (left) and ADAM7-transfected (right) cells described in panel A with no primary antibody. PD-3 (bottom), an ADAM7 monoclonal antibody, was unable to detect full-length ADAM7 on the cell surface of transfected (green line) cells as compared to controls (filled histogram). C) PD-10, an ADAM7 monoclonal antibody, was unable to detect a band corresponding to full-length ADAM7 in transfected and mock-transfected cells. Shown is a representative result for cell lines and hybridoma clones.

**Single Point-Mutation Bestows Catalytic Activity to a Noncatalytic ADAM**

We speculate that ADAM7 arose from a gene duplication event of ADAM28 and the gross structural integrity of the catalytic domain of noncatalytic ADAMs has been preserved throughout these evolutionary events. Indeed, noncatalytic ADAMs do not group together in a phylogenetic tree (Fig 11), lending credence to the idea that ADAM7 and the other noncatalytic ADAMs arose from multiple evolutionary events and not from a single common ancestor (Brocker, 2009). In light of our regulatory model (Fig 6), this would be an efficient way to mimic the molecular aspects of active counterparts but selectively eliminate the catalytic activity. To investigate this, we wanted to determine if restoration of the missing catalytic active site elements would "awaken" a dead protease.

The consensus glutamate within the ADAM7 catalytic active site was restored via a single point mutation (Fig 8). This was accomplished by substituting a single nucleotide, cytosine, within the glutamine codon (**C**AA) to guanine. Doing so provided the mutant ADAM7 with a fully restored consensus sequence within the metalloprotease domain. Recombinant Pro-Met Fc-fusion proteins (PM-Fc) for ADAM28, ADAM7 wt, and the active site ADAM7 $Q^{337}$/E mutant migrated at roughly 75kDa as expected when using reducing SDS-PAGE (Fig 12). These recombinant proteins exhibited sufficient purity to conduct the necessary proteolytic assays.

The α2M trapping assay was used to demonstrate catalytic activity of the ADAM7 active site mutant. α2M is a generic protease inhibitor that acts by covalently linking to the enzyme upon cleavage. This covalent linkage results in a higher molecular weight

species that can be observed by a gel shift. The presence of a higher molecular weight species is indicative of an active protease.

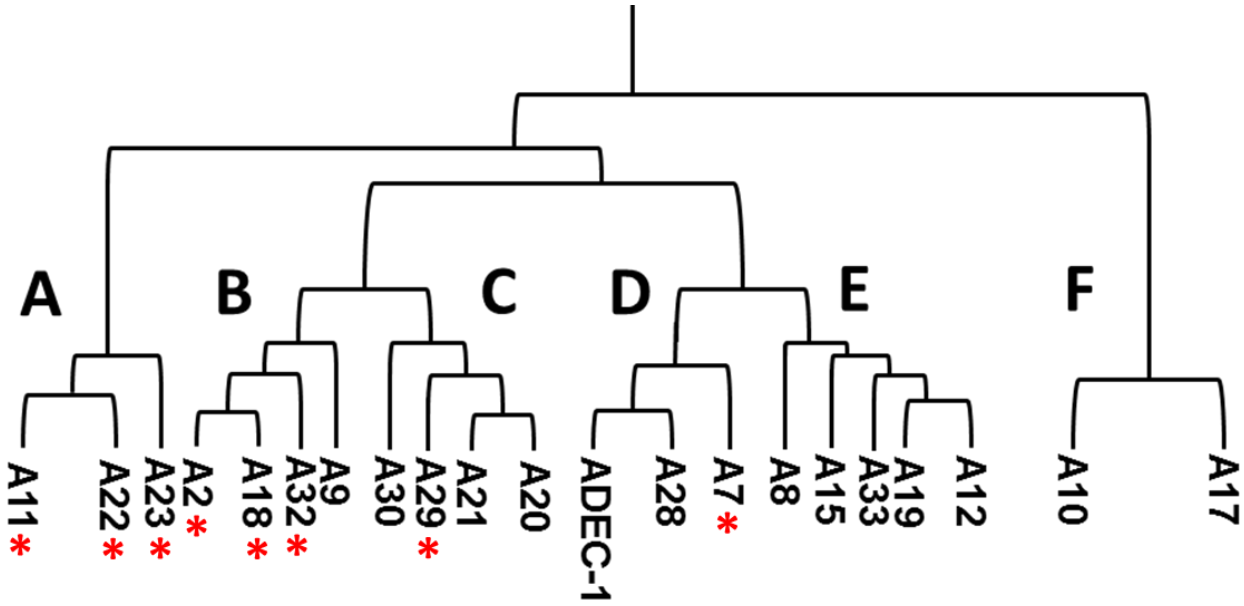

A
B
C
D
E
F
A11 *
A22 *
A23 *
A2 *
A18 *
A32 *
A9
A30
A29 *
A21
A20
ADEC-1
A28
A7 *
A8
A15
A33
A19
A12
A10
A17

**Figure 11. Phylogenetic Classification of Human ADAMs.** The 21 human ADAMs are grouped into six distinct clades based on phylogenetic analysis. Noncatalytic ADAMs (designated with a red asterisk) are grouped with catalytically active ADAMs in multiple clades, suggesting they arose from a common, catalytically active ancestor.

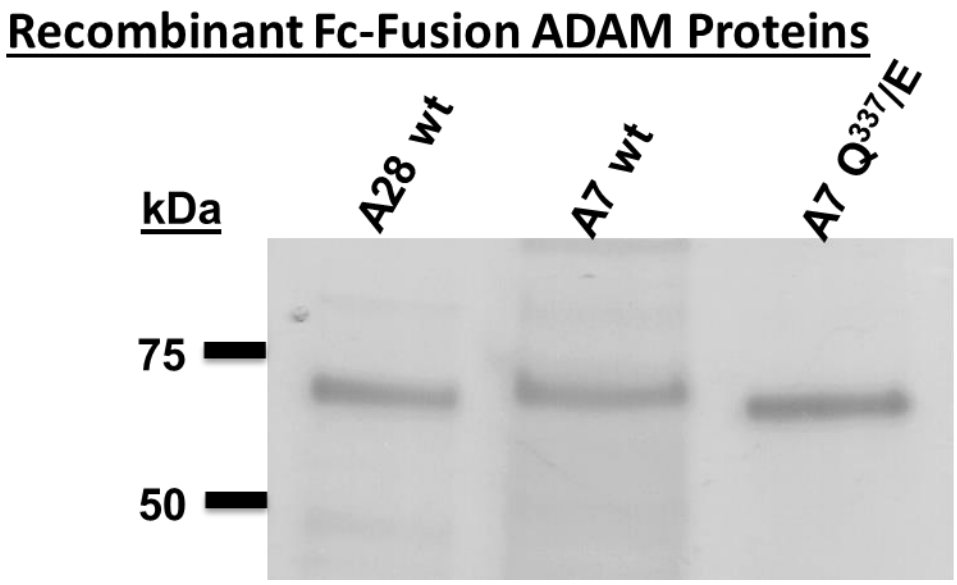

Recombinant Fc-Fusion ADAM Proteins
kDa
A28 wt
A7 wt
A7 Q337/E
75
50

**Figure 12. Purified Pro-Met Fc-Fusion Proteins.** Recombinantly produced and purified ADAM Pro-Met Fc-fusion proteins (3 μg/lane) were visualized by commassie staining. ADAM28 PM wt, ADAM7 PM wt, and ADAM7 PM $Q^{337}$/E are roughly 75 kDa as expected.

As expected, the positive control ADAM28 PM-Fc exhibited catalytic activity, whereas wild-type ADAM7 PM-Fc did not. Excitingly, and as anticipated, the ADAM7 $Q^{337}$/E mutant exhibited catalytic activity as evidenced by higher molecular weight species at 150kDa (Fig 13A). This is the first demonstration, to our knowledge, of a single point mutation within the active site bestowing catalytic activity to a noncatalytic ADAM.

**Effect of Glutamate and Zinc Concentration on Noncatalytic ADAM7**

While it appears that the catalytic activity exhibited by ADAM7 $Q^{337}$/E is the result of the mutation incorporated into the active site, the possibility existed that catalytic activity may result from other variables within the environment. Indeed, studies by M. Toney and J. Kirsch have demonstrated the ability to rescue enzymatic activity of an inactive mutant of aspartate aminotransferase simply through the addition of amines in solution (Kirsch, 1989; Kirsch, 1992).

If the catalytic function of noncatalytic ADAMs could be "activated" in localized environments, this could embody an unexplored aspect of how ADAM shedding activity is regulated, and suggest noncatalytic ADAMs are active *in vivo* given the right conditions. Specifically, glutamate and zinc within the environment were investigated for a possible role in giving rise to catalytic activity to a noncatalytic ADAM. Glutamate and zinc were selected due to their currently known roles in nucleophilic attack. Experimentally, the importance of zinc and glutamate for catalytic activity exhibited by ADAMs has been shown in many studies via the utilization of EDTA, a chelator of zinc, and E/A active site mutants in which glutamate was substituted for an alanine residue.

EDTA and the E/A active site mutants, separately, abolished catalytic activity that was previously exhibited by wild-type catalytically active ADAMs. (Gaultier, 2002; Smith and DeSimone, 2002; Chesneau, 2003)

The α2M trapping assay was modified to include increasing concentrations of glutamate and zinc (0-500μM). This concentration range was chosen as it covers the physiological values as well as supra-physiological levels that could occur in enriched environments or potential disease states. No catalytic activity was exhibited by wt ADAM7 PM-Fc at any concentration of zinc or glutamate tested (Fig 13B and 13C). These results demonstrate that the restored glutamate in the consensus active site of ADAM7 is essential for catalytic activity.

Additionally, these data indicate a potential preservation of the overall catalytic architecture in a noncatalytic ADAM including, but not limited to, metal ion coordination and spatial arrangement to facilitate nucleophilic attack when given the consensus active site residues.

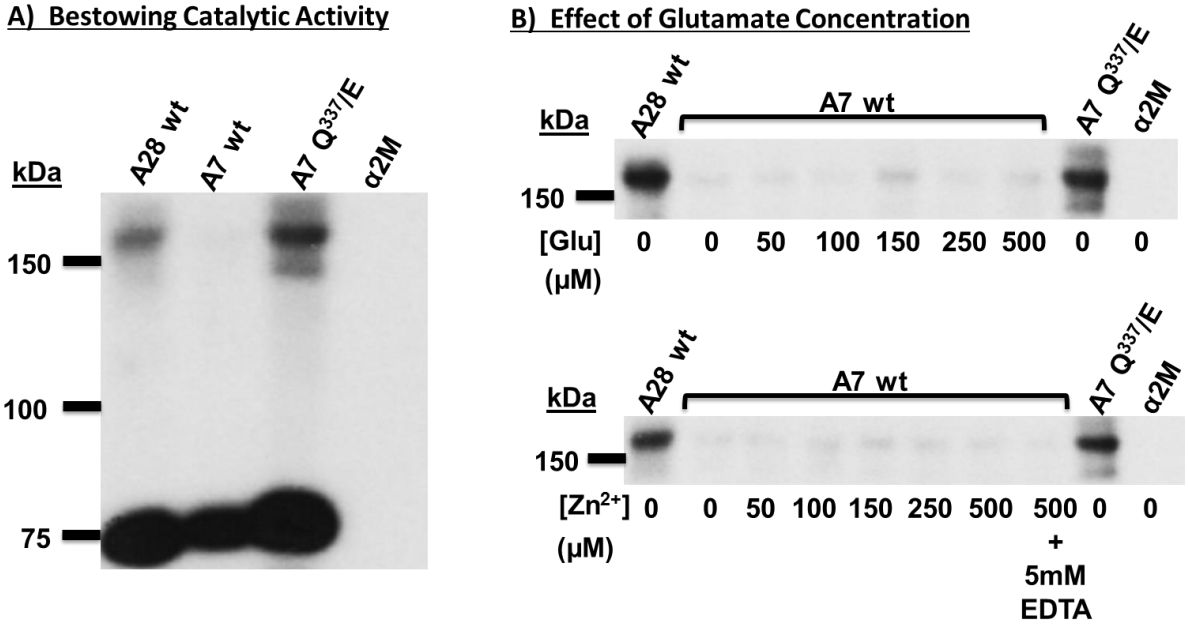

A) Bestowing Catalytic Activity
kDa
A28 wt
A7 wt
A7 Q337/E
α2M
150
100
75
B) Effect of Glutamate Concentration
kDa
A28 wt
A7 wt
A7 Q337/E
α2M
150
[Glu] (μM)
0 0 50 100 150 250 500 0 0
kDa
A28 wt
A7 wt
A7 Q337/E
α2M
150
[Zn2+] (μM)
0 0 50 100 150 250 500 500 + 5mM EDTA 0 0

**Figure 13. Restoration of Active Site Elements Bestows Catalytic Activity to Human ADAM7.** A) α2-Macroglobulin trapping assay demonstrates catalytic activity via gel-shift. ADAM28 PM-Fc wt and ADAM7 PM-Fc $Q^{337}$/E exhibit catalytic activity, while ADAM7 PM-Fc wt did not. B) Trapping assay from panel A were repeated with "dead" wt ADAM7 to determine if an enriched glutamate environment produces catalytic activity in noncatalytic ADAMs. C) The effect of zinc concentration on ADAM7 wt catalytic activity was determined. All images are representative of 3 independent runs.

## ADAM7 $Q^{337}$/E Exhibits Catalytic Specificity Mirroring ADAM28

The proposed gene duplication events that allowed ADAM7 to emerge as a potential regulator of ADAM28 likely maintained many properties and functions of ADAM28. Indeed, the integrin ligand properties of ADAM7 are identical to those of ADAM28 (Bridges, 2005; Edwards, 2008). As the ADAM7 mutant exhibited enzymatic activity, I wanted to determine if the catalytic properties specifically reflected those established for ADAM28 action. If ADAM7 could be "awakened" via mutations within the catalytic active site of the metalloprotease domain and exhibit catalytic properties that closely resembled those established for ADAM28, it would provide another functional link to the ADAM7 ancestry and provide additional information on the first known "awakened" noncatalytic enzyme.

ADAM28 cleaves myelin basic protein (MBP) at distinct sites (Howard, 2001). I modified this assay to address if ADAM7 would catalyze the hydrolysis of MBP in the same manner as previously established for ADAM28. Although other ADAMs, including ADAM10, are known to process MBP, each does so by producing unique cleavage products (Howard, 2001). Interestingly, ADAM7 $Q^{337}$/E activity towards MBP generated a similar fragment pattern as that obtained with the ADAM28 control (Fig 14). Because these data are qualitative, we are pursuing N-terminal sequencing of the respective fragments to further establish a functional link between ADAM7 and ADAM28. By demonstrating overlap in substrate specificity between ADAM28 and ADAM7, these results support our model that ADAM28 and ADAM7 are evolutionarily and functionally linked.

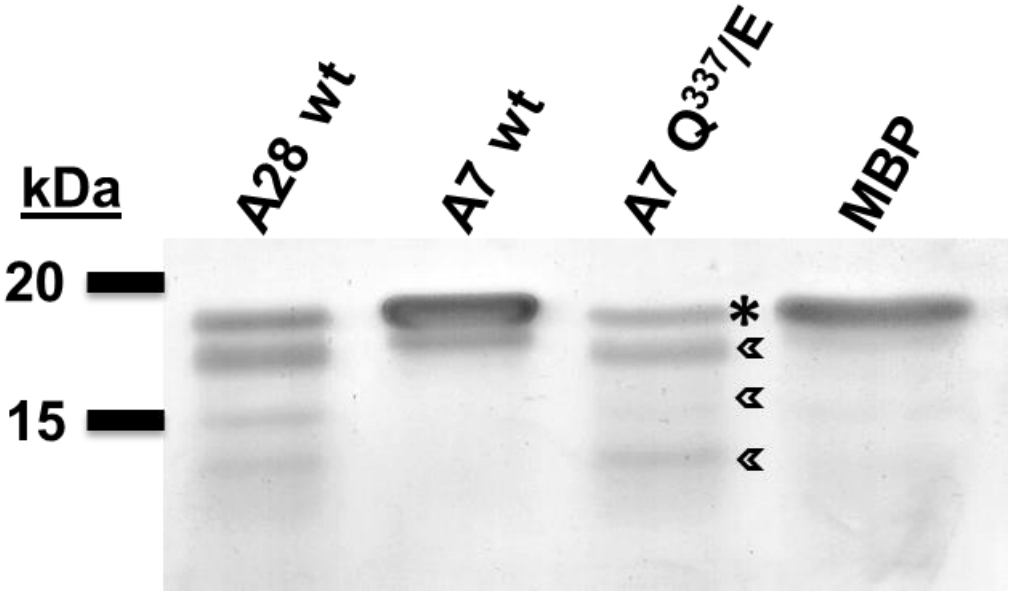

kDa
A28 wt
A7 wt
A7 Q337/E
MBP
20
15
*

**Figure 14. Catalytic Specificity of ADAM7 $Q^{337}$/E.** Myelin basic protein (MBP) cleavage assays demonstrate that the ADAM7 active site mutant qualitatively exhibits catalytic specificity mirroring ADAM28 wt. Full-length, unprocessed MBP (~18 kDa) is denoted by the asterisks. Arrows indicate MBP cleavage products.

## Discussion

ADAMs are a family of proteases that have been implicated in many aspects of human health and disease through catalyzing ectodomain shedding, a process in which cell-surface substrates are liberated into soluble, biologically active derivatives. While ADAM-mediated shedding is critical to the normal health state, including cardiac development (Zhou, 2004) and immune response (Gooz, 2010), dysregulation of ADAM-mediated shedding has been implicated in many pathological conditions, such as cardiac hypertrophy (Asakura, 2002) and chronic inflammatory states (Klein, 2010; Bzowska, 2004). A better understanding of how ADAM-mediated ectodomain shedding is regulated could provide new therapeutic avenues for the deleterious conditions associated with abnormal ADAM activity. A summary of known regulatory mechanisms of ADAM proteolytic action is available in Table 1. Largely, these mechanisms are standard dogma to the field of enzymology. Textbooks classically categorize regulation into inhibition, zymogen production, genetic control, feedback/feedforward allosteric control, and covalent modification (e.g. phosphorylation), and current knowledge regarding ADAM regulation does not greatly differ from these previously established mechanisms.

My work is aimed at providing initial support for a previously unexplored regulatory mechanism involving noncatalytic ADAMs. Noncatalytic ADAMs lack one or more consensus elements within the catalytic active site leading to a “dead” enzyme. Despite being noncatalytic, they have retained the complete domain architecture of their active counterparts (Liu, 2009), and exhibit functional activity (e.g. integrin binding) that is attributed to these specific nonenzymatic domains (Edwards, 2008). My proposed

mechanism of noncatalytic ADAMs governing the availability of substrates could potentially alter the way enzyme regulation is viewed and open new therapeutic avenues for the treatment of pathologies associated with ADAM-mediated shedding by providing a new drug target, the noncatalytic ADAM. To be feasible, this novel regulatory mechanism requires expression of noncatalytic ADAMs within the same niche as catalytically active counterparts to produce competition for binding partners. Through competition with catalytically active ADAMs, these noncatalytic ADAMs may effectively regulate the activity of their catalytically active counterparts by governing substrate availability or localization to the substrate through other molecular interactions, namely integrin binding.

In the current proposal, ADAM7 and ADAM28 were used as a model system to initiate studies addressing the validity of our regulatory model. ADAM28 is catalytically active and has been to play a role in various biological processes from transmigration of lymphocytes (McGinn, 2011) to TNF-α shedding (Jowett, 2012). ADAM7 is noncatalytic, but is suggested to play a role in increased metastasis of melanoma cells (Wei, 2011). ADAM28 and ADAM7 were chosen as my model pair due to the striking similarity of their disintegrin domain protein sequence and common binding partners, as well as the the possibility that ADAM7 may have arisen from ADAM28 via evolutionary events (Bates, 2002). Initially, I set out to determine the expression patterns of ADAM7 at the protein level utilizing ADAM7 monoclonal antibodies our lab had developed against the ADAM7 disintegrin domain. Unfortunately, these antibodies did not recognize full-length ADAM7 exogenously expressed on the surface of multiple cell lines under the conditions tested. This was confirmed in flow cytometry with intact cells

as well as with reduced and denatured isolated cell-surface protein via immunoblot. At this point, it still remains to be determined if ADAM7 is expressed within the same cellular niche as ADAM28 in primary human cells or cell lines. Our ADAM7 monoclonal antibodies are still in ascites, which is the fluid produced in the peritoneal cavity during production of the antibodies. Future directions are to purify the antibodies from the ascites stage, as to reduce any potential contaminants in the supernatant, for a reduction in cross-reactivity and subsequent increased sensitivity to full-length ADAM7 disintegrin domain.

The second part of my thesis used ADAM7 to determine the magnitude of metalloprotease domain preservation and similarity to ADAM28. An active site mutant, ADAM7 $Q^{337}$/E, was developed in which the active site consensus element, glutamate, was restored via a single point mutation. The restoration of glutamate provided ADAM7 with an intact consensus sequence (HExxHxxGxxH) within the active site.

Utilizing an α2M trapping assay, ADAM7 $Q^{337}$/E was shown to exhibit catalytic activity, providing the first demonstration to my knowledge of “awakening” a dead protease through a single point mutation within the catalytic active site. This was a very exciting result as it also provided the first evidence to suggest the gross structural preservation of the catalytic active site in the metalloprotease domain of noncatalytic ADAMs. As only a single amino acid within the active site was altered, the data suggests the overall structural elements that facilitate substrate interactions were present and intact with the noncatalytic ADAM7. Additionally, these data revealed for the first time that a noncatalytic ADAM’s metalloprotease domain has the ability to

interact known ADAM substrates, a key component of the proposed regulatory mechanism.

Based upon the literature, it was feasible that increased concentrations of zinc or glutamate might account for the observed catalytic activity of ADAM7 (Kirsch, 1989; Kirsch 1992). If wild type ADAM7 encounters environments enriched for zinc or glutamate *in vivo*, conditions could be favorable enough to induce ADAM7 to exhibit catalytic activity. This concept embodies another potential regulatory mechanism of noncatalytic ADAMs. In fact, it would suggest that noncatalytic ADAMs are indeed active in select circumstances. However, upon modification of the α2M assay to include increasing concentrations of glutamate and $Zn^{2+}$, catalytic activity was not exhibited by wild-type ADAM7. These results further confirmed the role of active site consensus elements, particularly glutamate, and the effects of their restoration in a noncatalytic ADAM.

Following the demonstration of general catalytic activity, I then examined the catalytic specificity exhibited by ADAM7 $Q^{337}$/E. Specificity of interactions between the metalloprotease domain and substrates provides crucial insight to further validating the proposed regulatory mechanism, as it may suggest the ability of a noncatalytic ADAM to interact with substrates via the metalloprotease domain. Interaction in this manner between noncatalytic ADAMs and substrates, while not producing cleavage products, may still provide a means of competitive binding.

Catalytic specificity of ADAM-mediated shedding was established using myelin basic protein (MBP), a well-known substrate of ADAM28 and other ADAMs (Howard,

2001). As shown in figure 12, ADAM7 $Q^{337}$/E appears to produce cleavage products of similar size to those produced by ADAM28, suggesting a similarity in catalytic specificity.

These results, as well as the preliminary data also discussed, have provided key evidence to support the potential for a regulatory mechanism in which noncatalytic ADAMs govern the availability of substrates in the presence of their catalytically active counterparts through competitive binding. Additionally, by demonstrating previously unknown functions of noncatalytic ADAMs, these data have potentially opened the door to new areas of investigation:

***i)*** **How enzymatic regulation is viewed.** As previously discussed, the known regulatory mechanisms are centralized around active enzymes and substrates. However, now that a novel regulatory role involving noncatalytic ADAMs has been proposed and supported, investigation into the putative regulatory roles of other noncatalytic enzymes is merited. One example, phospholipase C-related catalytically inactive protein (PRIP) has actually been shown to regulate lypolytic activity of hormone-sensitive lipase in adipose tissue via phosphatase translocation (Okumura, 2014). While this is not competitive binding of substrate, it still represents a regulatory mechanism in which a noncatalytic enzyme is regulating the activity of a catalytically active enzyme expressed within the same niche.

***ii)*** **Restoration of catalytic activity.** A single point mutation bestowed catalytic activity to ADAM7. This is a very novel observation and it remains to be determined if restoration of consensus elements within the active site can bestow catalytic activity to

other noncatalytic ADAMs. While some noncatalytic ADAMs (ADAM7, ADAM29) are only lacking a single consensus element within the active site, others are lacking several (Wei, 2011). In fact, ADAM11 has retained no recognizable consensus elements (Table 2). Restoration of the elements in ADAM11, or another protease that may be lacking all required elements, would begin to answer how far removed these "dead" enzymes are from catalytic activity. The ability to reproduce the effect observed with ADAM7 in another ADAM, especially one lacking many or all consensus elements, would provide further details regarding the preservation of the metalloprotease domain throughout evolutionary events. Due to the presence of noncatalytic enzymes in multiple classes, the impact of exploring this feature of "raising the dead" has potentially broad implications across multiple disciplines.

## Conclusion

In summary, I have demonstrated preservation of the gross structural integrity of the metalloprotease domain of noncatalytic ADAMs. In doing so, the “awakened” ADAM7 displayed catalytic specificity qualitatively mirroring that of ADAM28. Despite the inability to determine ADAM7 expression at the protein level, these results provide key data to support the existence of a role for noncatalytic ADAMs in regulating the shedding activity of their catalytically active counterparts by governing substrate accessibility and availability.

## Future Directions

Many follow-up experiments to the findings in my thesis will be pursued to provide additional evidence of our regulatory model. With respect to the "walking dead" ADAM7 active site mutant, several approaches will be undertaken to provide additional data to my exciting preliminary findings. Excising and analyzing the MBP cleavage products via N-terminal sequencing and mass spectrometry to determine the specific cleavage sites will provide definitive evidence of catalytic specificity of the ADAM7 mutant as compared to ADAM28.

To further assess the structural similarities of the metalloprotease domain catalytic active site of ADAM7 and ADAM28, a series of modified α2M assays will be used. Confirmation of catalytic activity of ADAM7 $Q^{337}$/E via metalloprotease components will be determined using broad spectrum inhibitors. Among those to be used are chelators, EDTA/EGTA and 1,10-phenanthroline, as well as inhibitors of various protease types, such as Aprotinin, PMSF, Pepstatin A, Pefabloc-SC, and Leupeptin. We expect only the chelators to abolish catalytic activity. To verify this, and that they are working through chelation as opposed to competitive binding, $Zn^{2+}$ will be added in excess in the presence of the chelators to rescue catalytic activity. A pitfall of this is the single aspartate residue following the final histidine within the consensus sequence of the catalytic active site, which could potentially play a role in catalytic activity, as occurs via aspartate proteases. This will be determined using Pepstatin A, which is an inhibitor of aspartate proteases.

Following use of the broad spectrum inhibitors, a series of physiologically relevant metalloprotease inhibitors will be used. Tissue Inhibitor of Metalloproteases (TIMPs) are a family of metalloprotease inhibitors used extracellularly to regulate the activity of Matrix Metalloproteases (MMPs) and ADAMs to maintain a homeostatic environment. Four members of the TIMP family are known, with three (TIMP-1, TIMP-3, and TIMP-4) displaying activity toward the ADAMs. TIMPs abolish catalytic activity in a two-step inhibition mechanism in which they chelate metal cations, followed by competitive binding within the catalytic active site. The use of TIMP-1, TIMP-2, and TIMP-3 in a modified α2M trapping assay with ADAM28 and ADAM7 $Q^{337}$/E will further assess catalytic activity resulting from metalloprotease components, as well as potential catalytic active site similarity between ADAM7 and ADAM28. We expect ADAM7 $Q^{337}$/E to be inhibited by TIMP-3, but not TIMP-1 and TIMP-2, as that is the established pattern of inhibition of ADAM28 via TIMPs. Pitfalls of this study include the inability of TIMP-3 to inhibit ADAM7 $Q^{337}$/E, as well as inhibition of the awakened ADAM7 mutant by TIMP-1 and/or TIMP-2. Differences of inhibition patterns would demonstrate a structural difference among the catalytic active site and metalloprotease domain of ADAM7 and ADAM28, however we do not expect that to occur.

Additionally, the ADAM7 active site mutant and ADAM28 will be used in a series of assays designed to investigate catalytic efficiency and binding affinity via the metalloprotease domain with significant contributions from Dr. Tonya Zeczycki. Obtaining kinetic measurements of the ADAM7 mutant to compare with the kinetic properties of ADAM28 will provide further evidence as to the similarity of the catalytic active site in the metalloprotease domain of catalytically active and noncatalytic ADAMs.

Determining binding affinity ($K_D$) of these enzymes would provide significant insight into how the potential regulation occurs. It is unknown how much noncatalytic ADAM would need to be expressed to yield an inhibitory effect. This is likely to be contingent on how avid the noncatalytic ADAMs bind substrate and/or receptors as compared to catalytic counterparts. The ADAM7 mutant characterized here would allow for such approaches. In addition, a soluble cell-binding assay to investigate integrin binding kinetics would be worthwhile. For determination of the $K_D$ of ADAM7 and ADAM28 for integrin receptors, a modified version of the assay shown in Figure 5 will be run in which fluorescently conjugated ADAM ligands are used to generate standard curves correlating protein quantity to fluorescent intensity. Unlabeled competitors (ADAM7), as well as noncompetitive controls such as ADAM29, will be titrated in for determining competitive inhibition ($K_i$).

The ability of a noncatalytic ADAM to competitively inhibit a catalytically active counterpart will be assessed using a breast cancer cell line, MDA-MB231. ADAM28 cleavage of insulin-like growth factor binding protein-3 (IGFBP-3) into distinguishable products is detectable using established reagents in various techniques. Exogenous ADAM7 expression is expected to decrease ADAM28-mediated shedding of IGFBP-3 via competitive inhibition. These expected results would provide further evidence indicating the ability of a noncatalytic ADAM to competitively bind substrate in the presence of a catalytically active counterpart. Additionally, exogenous expression of ADAM7 $Q^{337}$/E is expected to increase IGFBP-3 shedding, which would be indicative of an overlap in substrate recognition and, potentially, catalytic specificity.

Next, it would be crucial to identify expression patterns of ADAM7 at the protein level. As previously mentioned, purification of the hybridoma supernatant may be required to reduce cross-reactivity and increase selectivity toward ADAM7. Optimization of techniques, including flow cytometry and immunohistochemistry, would provide multiple avenues of ADAM7 detection on the cell surface. Upon establishing ADAM7 expression at the cell surface, ADAM28 expression should then be confirmed. The expression patterns, along with the kinetic studies, would provide necessary data to confirm the existence of a regulatory model involving noncatalytic ADAMs.

Finally, outside of the proposed regulatory role, phylogenetic analyses of noncatalytic ADAM metalloprotease domain active sites would address the question if these ADAMs truly arose from their catalytically active counterparts or if they actually arose from a single, noncatalytic ancestor. For this, Dr. Tyra Wolfsberg, of the National Human Genome Research Institute at NIH, has agreed to collaborate with our lab to conduct these studies. With Dr. Wolfsberg's assistance, the knowledge gap surrounding noncatalytic ADAMs could be closed significantly.

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