
**ANNELIZE ZAMBON BARBOSA ARAGÃO**

# A TIOREDOXINA-1 É UMA NOVA PARCEIRA DE INTERAÇÃO DO DOMÍNIO CITOPLASMÁTICO DA ADAM17 E PARTICIPA DA SUA MODULAÇÃO

# *THIOREDOXIN-1 IS A NOVEL LIGAND OF ADAM17 CYTOPLASMIC DOMAIN AND PARTICIPATES IN ITS MODULATION*

**CAMPINAS**
**2014**

UNIVERSIDADE ESTADUAL DE CAMPINAS
Instituto de Biologia

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## *THIOREDOXIN-1 IS A NOVEL LIGAND OF ADAM17 CYTOPLASMIC DOMAIN AND PARTICIPATES IN ITS MODULATION*

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas, como parte dos requisitos exigidos para obtenção do título de Doutora em Biologia Funcional e Molecular, na área de Bioquímica.

*Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Functional and Molecular Biology, in Biochemistry.*

**Orientadora/*Supervisor*: Dra. Adriana Franco Paes Leme**

**ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA ANNELIZE ZAMBON BARBOSA ARAGÃO E ORIENTADA PELA DRA. ADRIANA FRANCO PAES LEME.**

Adriana Franco Paes Leme

**CAMPINAS**

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Ana Marria Rabetti - CRB 8/2471

Ar12t

Aragão, Annelize Zambon Barbosa, 1984-
A Tioredoxina-1 é uma nova parceira de interação do domínio citoplasmático da ADAM17 e participa da sua modulação / Annelize Zambon Barbosa Aragão. – Campinas, SP : [s.n.], 2014.

Orientador: Adriana Franco Paes Leme.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Proteinas. 2. Espectrometria de massas. I. Leme, Adriana Franco Paes. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações para Biblioteca Digital

**Título em outro idioma:** Thioredoxin-1 is a novel ligand of ADAM17 cytoplasmic domain and participates in its modulation
**Palavras-chave em inglês:**
Proteins
Mass spectrometry
**Área de concentração:** Bioquímica
**Titulação:** Doutora em Biologia Funcional e Molecular
**Banca examinadora:**
Adriana Franco Paes Leme [Orientador]
Jörg Kobarg
Carmen Veríssima Ferreira Halder
Solange Maria de Toledo Serrano
Alexandre Keiji Tashima
**Data de defesa:** 01-07-2014
**Programa de Pós-Graduação:** Biologia Funcional e Molecular

Campinas, 01 de julho de 2014

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## RESUMO

A metaloprotease ADAM17 é uma das mais importantes reguladoras dos mecanismos de sinalização celular, pois é responsável pela liberação de ectodomínios de proteínas de superfície participando da regulação de processos fisiológicos, mas também tem sido associada à progressão de tumores e processos inflamatórios. Este estudo buscou entender a regulação da atividade proteolítica da ADAM17 por meio do seu domínio citoplasmático. Para isso, foi utilizada uma abordagem de co-imunoprecipitação seguida por análise dos parceiros de interação por espectrometria de massas. Dentre os parceiros identificados, a tioredoxina-1 (Trx-1) foi o alvo escolhido e a interação com o domínio citoplasmático da ADAM17 foi validada em experimentos complementares de microscopia confocal, ensaio de ligação em fase sólida, ressonância magnética nuclear e determinação da interface de interação por ligação química seguida de análise por espectrometria de massas. Experimentos funcionais para entender o papel funcional dessa interação foram realizados e é interessante notar que a superexpressão de Trx-1 diminui a atividade proteolítica da ADAM17 sobre o substrato HB-EGF, resultado obtido utilizando metodologia baseada em cultura de células. A descoberta de um novo parceiro de interação da ADAM17, capaz de modular a sua ativação permitirá que novos trabalhos sejam realizados para entender o mecanismo pelo qual essa metaloprotease atua.



## ABSTRACT

ADAM17 is a metalloprotease which plays an important role in regulatory mechanisms of cell signaling. It is responsible for shedding of the surface proteins, participating in regulation of important physiological processes, but it has also been associated with cancer progression and inflammatory processes. The aim of this study was to explore the regulation of ADAM17 by its cytoplasmic domain. In this regard, we used an approach based on co-immunoprecipitation followed by mass spectrometry (MS). Among the identified partners, the thioredoxin-1 (Trx-1) was selected, and its interaction has been validated using confocal microscopy, solid-phase binding assay, nuclear magnetic resonance and chemical cross-linking followed by MS. To understand the functional role of this interaction, experiments using cell-based assay were performed. Trx-1 overexpression decreases HB-EGF shedding upon ADAM17 activation. The discovery of a new interaction partner of ADAM17, which can modulate its activation, gives insights for novel studies to understand the mechanism of action of this metalloprotease.

## SUMÁRIO

Às duas pequenas companheiras que, mesmo sendo caninas, me ensinaram a ser mais humana.

**“Os muros gretados são muito mais belos que os muros lisos.”**

**Mário Quintana**

**AGRADECIMENTOS**

Obrigada aos amigos que fizeram dos muros lisos, gretados e deixaram mais belos os dias onde nem tudo foi perfeito; nem todo experimento, um sucesso; nem todo resultado, coerente e nem todo esforço, recompensado.

Dentro e fora do laboratório, a presença de vocês deixou os obstáculos menores, fazendo dos muros, muretas. Todos do grupo MAS, especialmente: Fernando, Sami, Rebeca, Daniela, Frederico, Romênia, Bianca, Flávia W., Flávia Z., Mariana F., Renato, Mariana Z. e Marília. Dos outros grupos: Ângela, Rafael, Melissa, Malu, Rodrigo, Bárbara, Zaira, Ariane, Edmárcia e tantos outros. De Piracicaba (FOP): Andreia, Michelle, Isadora e todas as meninas que me receberam por lá tão bem. De fora do lab (mas não menos importantes): Rebeca, Marina, Elias, Fábio, Fernanda, Ana Carolina L., Ana Carolina T., André, Tais, Haira, Bruno, Ricardo, Ana, Natália e outros mais, de perto e de longe.

Obrigada aos meus amores, minha família: mãe (Tere), irmã (Thayse), cunhado (Simba), tios, primos, avó e namorado (Lucas). Quanto mais liso o muro, maior foi o incentivo de vocês para que eu pudesse colori-lo. Nada teria sentido se não fosse por vocês.

Obrigada aos professores/pesquisadores que nos momentos onde me faltaram cores estavam dispostos a emprestar suas próprias tintas: Jörg Kobarg, Kleber Franchini, Sandra Dias, Ana C M Zeri, Celso Bendetti, Marcio Chain, Paulo Oliveira, Tomomasa Yano, Hernandes Carvalho, Sara T O Saad, Carmen V Ferreira, Francisco Laurindo, João E Soares Netto, Ricardo Della Coleta. *Thanks to Nick Sherman and J. Fox for the special attention with me and this study*.

E se os professores emprestaram as tintas, os técnicos de cada um deles me emprestaram ferramentas e ensinaram como usá-las. Quero agradecer novamente e especialmente à Sami, Bianca e Romênia, mas também à Thais, Maria Eugênia, Wanderley, Carol, Jaqueline, Renata e tantos com quem pude conviver nesses anos, excelentes profissionais que me inspiram nessa nova etapa da minha vida.

Preciso deixar registrado um agradecimento especial aos alunos e colegas de trabalho que me acolheram quando cheguei ao IQ-UNICAMP, obrigada pela compreensão nessa fase de redação da tese, exame de qualificação e preparação para

a defesa. Carlos Ramos, Pimpim, Aline, Vanessa, Mariana, Gláucia, Josielle, David, Clelton, Regina, Bárbara, Conrado, Letícia, Claudinha, Natália, João, Ingrid, Nayene, muito obrigada pelo apoio.

Não posso deixar de agradecer também às pessoas que muitas vezes não são lembradas, ou passam despercebidas: porteiros, jardineiros, seguranças, faxineiras, pessoal da manutenção... Sem eles nada estaria funcionando tão bem e o *show* não poderia continuar. Obrigada!

Quero agradecer imensamente e especialmente à minha orientadora, Adriana Paes Leme, pela disposição, positividade, compreensão, apoio, confiança e sobretudo, por me fazer acreditar que não há muro tão alto que não possa ser ultrapassado.

À Fapesp por ter permitido que eu me dedicasse integralmente, durante 3 anos, a esta pesquisa e às instituições que tornaram todo o trabalho possível: CNPEM, LNBio e UNICAMP.

## LISTA DE ILUSTRAÇÕES

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**Capítulo 1 "*Identification of Novel Interaction Between a Disintegrin and Metalloprotease-17 (ADAM17) and Thioredoxin-1*"**

# 1 INTRODUÇÃO

## 1.1 ADAMs

As ADAMs (*A Disintegrin And Metaloprotease*) são as metaloproteases de mamíferos, pertencentes à família das adamlisinas, responsáveis por clivar ectodomínios de proteínas de superfície celular, liberando-os para o meio extracelular. Devido ao seu papel fundamental, as ADAMs são consideradas enzimas-chave de diferentes vias regulatórias, atuando em condições de homeostase (estado fisiológico) ou desequilíbrio (estado patológico). A desregulação dessas proteases tem sido associada a algumas doenças, como câncer, problemas cardiovasculares, asma, processos inflamatórios, Doença de *Alzheimer*, entre outras (EDWARDS *et al*., 2008).

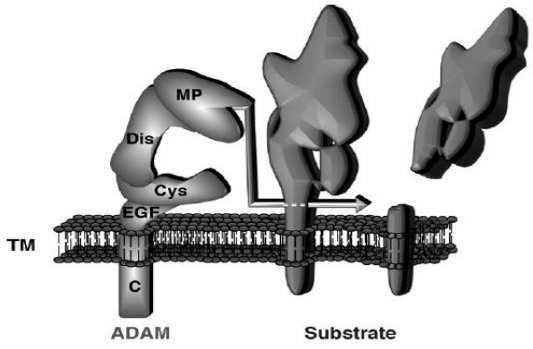


**FIGURA 1: Esquema da estrutura dos domínios das ADAMs e o processo de clivagem de substratos.** Ectodomínio: MP (domínio metaloprotease); Dis (domínio tipo-disintegrina); Cys (domínio rico em cisteínas); EGF (domínio tipo-fator de crescimento epidermal); TM (domínio transmembrana) e C (domínio citoplasmático). Adaptado de REISS & SAFTIG, 2009.

As ADAMs são proteínas transmembrana, formadas por uma complexa estrutura de multidomínios (FIGURA 1). Em geral, essas proteases apresentam uma sequência sinal N-terminal que direciona a proteína recém traduzida para o sistema de secreção celular. A passagem pelo Complexo de Golgi é fundamental para que a ADAM receba algumas das modificações pós-traducionais (glicosilações) necessárias. Após o transporte, a enzima é endereçada para a membrana em sua forma inativa (zimogênio) em função da presença de um pró-domínio, que funciona como uma chaperona intramolecular. Muito tem se especulado em relação à remoção do pró-domínio, mas acredita-se que convertases, como a furina, sejam as proteínas responsáveis por esse processamento (MILLA *et al.*, 1999; SCHLONDORFF *et al.*, 2000; GONZALES *et al.*, 2004; BUCKLEY *et al.*, 2005). Uma vez removido o pro-domínio, a forma madura da proteína está apta a realizar sua atividade catalítica, através do domínio metaloprotease, que é coordenado por um íon metálico, ($Zn^{2+}$ no caso das ADAMs). O domínio metaloprotease é o responsável pela atividade da enzima, por isso também é chamado de domínio catalítico, capaz de regular os processos fisiológicos por promover a liberação de ectodomínios de moléculas sinalizadoras (SEALS & COURTNEIDGE, 2003). Dentre os principais alvos das ADAMs estão citocinas, fatores de crescimento, seus receptores e moléculas de adesão, desencadeando uma série de vias de sinalização, adesão, migração e proteólise (MURPHY, 2008).

Adjacentes ao domínio catalítico estão os domínios tipo-disintegrina, que foi assim denominado por sua afinidade e ligação à integrinas, o domínio rico em cisteínas e o domínio tipo-EGF que ainda têm seu papel pouco conhecido, mas propõe-se que estejam

relacionados à ligação com determinados substratos, permitindo sua clivagem de um modo específico. Em conjunto, todos esses domínios formam o ectodomínio e estão voltados para o exterior da célula.

Devido à presença desses domínios típicos, as ADAMs têm relação com outras metaloenzimas como as metaloproteases solúveis (ADAM-TS) que apresentam também um domínio trombospondina, as metaloproteases de matriz extracelular (MMPs) e as metaloproteases de veneno de serpente (SVMPs) (FOX & SERRANO, 2008) (FIGURA 2).

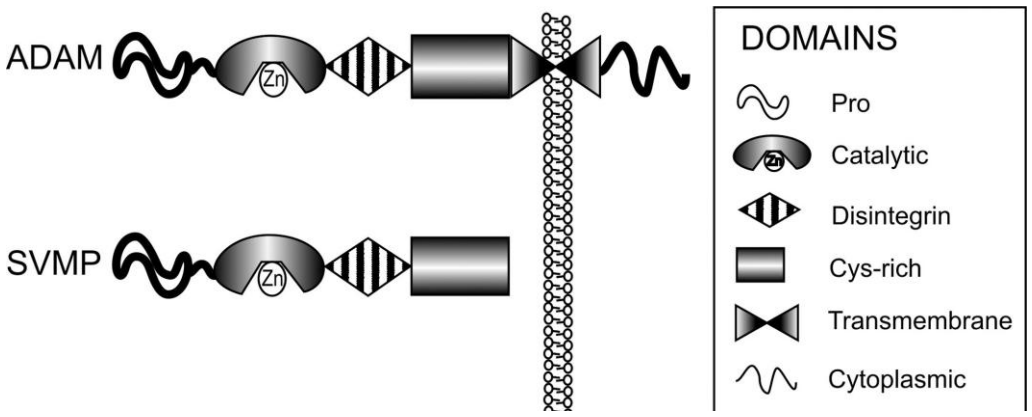


**FIGURA 2: Similaridade de domínios entre ADAM e classe PIII de SVMP (*snake venon metalloprotease*).** A organização dos domínios é comparada, mostrando que ambas apresentam o pró-domínio seguido do domínio catalítico dependente de ligação ao $Zn^{2+}$, domínio tipo-disintegrina e rico em cisteínas. Somente as ADAMs apresentam o domínio transmembrana e citoplasmático, que permitem sua ancoragem à membrana. Adaptado de GOOZ, 2010.

As ADAMs também apresentam uma sequência chamada domínio citoplasmático, ou cauda citoplasmática, voltada para o interior da célula. Apesar do domínio catalítico ser bastante conservado entre os membros dessa família, o domínio citoplasmático é o mais variável. São apenas 11 resíduos de aminoácidos para ADAM11, 197 resíduos para ADAM13 e 131 resíduos para a ADAM17, por exemplo. Esses domínios apresentam algumas regiões similares, sugerindo ter funções conservadas. O domínio citoplasmático é rico em resíduos de serina e prolina, contendo sequências consenso RXXPXXP e PXXPXR para interação com domínios Src (SH3) de outras proteínas (FENG *et al*., 1994). Alguns domínios citoplasmáticos contêm resíduos de tirosina, treonina e serina, que podem ser substratos para quinases, e quando fosforilados podem atrair ou repulsar certos ligantes (PAWSON & NASH, 2000). Essas interações podem estar relacionadas à função do domínio citoplasmático na regulação do domínio catalítico através da regulação do tipo *inside-out* (de dentro para fora). Além dessa função, o domínio citoplasmático têm sido sugerido como responsável pela maturação e localização subcelular (BLOBEL, 2000) das ADAMs. A fosforilação de resíduos no domínio citoplasmático tem sido associada com a atividade das ADAMs, visto que POGHOSYAN *et al*. (2002) mostraram que a desfosforilação do extrato celular resultou na diminuição da associação da ADAM15 com seus ligantes. Assim, as características observadas no domínio citoplasmático sugerem seu papel singular na sinalização intracelular e o conhecimento das proteínas que interagem com esse domínio pode ajudar a elucidar o papel dessas enzimas no desenvolvimento do câncer, inflamação ou ainda na manutenção da homeostase. O papel do domínio

citoplasmático na sinalização intracelular, refletida na própria atividade da ADAM, como também nas vias que essa protease pode regular precisa ser completamente esclarecido.

Outros mecanismos também são sugeridos para a regulação das ADAMs. Essas metaloproteases parecem ser ativadas principalmente por sistemas de mensageiros secundários, como $Ca^{2+}$ intracelular e proteína quinase C (PKC), e pelas vias de MAPK (*mitogen activated protein kinase*) (DÍAZ-RODRÍGUEZ *et al.*, 2002) (FIGURA 3). As três principais vias de MAPK em células de mamífero são JNK, p38 e ERK1/2. Porém, essas vias não são as únicas descritas para a ativação das ADAMs. As espécies reativas de oxigênio (ROS) também podem atuar na modulação do estado oxidativo das cisteínas das ADAMs e alterar sua atividade sobre alguns substratos (WANG *et al.*, 2009). No entanto, a maneira como ocorre essa modulação e se existem outros mecanismos envolvidos permanecem desconhecidos.

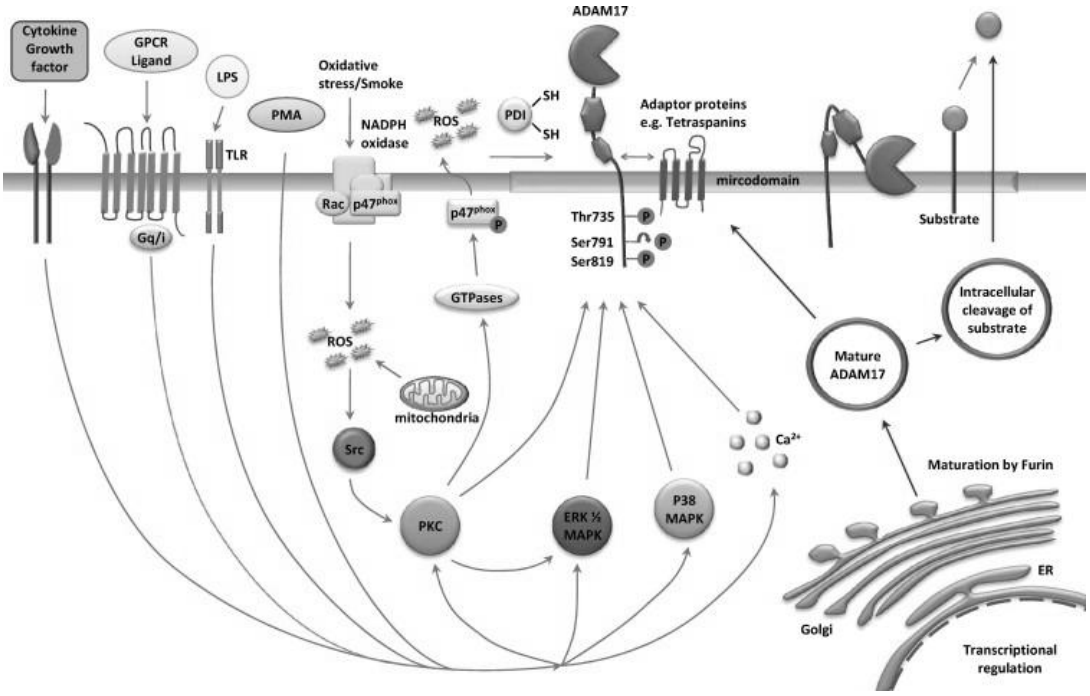


**FIGURA 3: Vias de ativação das ADAMs.** Fatores de crescimento, agonistas de GPCR, ligantes de TLR (*toll-like receptors*) (LPS), ésteres de forbol (PMA), espécies reativas de oxigênio (ROS), MAPKs (p38 e ERK1/2), mensageiros secundários ($Ca^{2+}$ e PKCs) e isomerases de grupos tiol (PDI) são algumas das moléculas responsáveis pela ativação das ADAMs. Retirado de DREYMUELLER *et al.*, 2012.

Em resumo, a atividade proteolítica da ADAM17 tem sido associada ao desenvolvimento e progressão de tumores, à proteólise de componentes da matriz extracelular, à liberação de proteínas de superfície e sinalização via integrinas (ROCKS *et al*., 2008; MURPHY, 2008). Entretanto, o papel e a identidade dos ligantes do domínio citoplasmático, associado ao mecanismo pelo qual eles respondem pela ativação das ADAMs, ainda não foram elucidados.

## 1.2 ADAM17

A ADAM17 é o membro da família das ADAMs melhor estudado até o presente momento. Foi descoberta em 1997, sendo descrita simultaneamente por dois grupos de pesquisa em artigos na revista *Nature* (BLACK *et al.*, 1997; MOSS *et al.*, 1997). Essa protease também recebe o nome de TACE (TNF-α *converting enzyme*) por ser a principal responsável pela conversão do proTNF-α em sua forma solúvel.

De acordo com o banco de dados do UniProt (número de acesso P78536), a ADAM17 humana é composta por 824 resíduos de aminoácidos, que compreendem os seguintes domínios e/ou motivos: 1-17 – sequência sinal; 18-214 – pró-domínio; 215-671 – ectodomínio (sendo os resíduos 223-474 – domínio metaloprotease; 475-563 domínio tipo-disintegrina; 564-602 domínio rico em cisteínas; 603-671 – domínio tipo-EGF); 672-692 – domínio transmembrana; 693-824 – domínio citoplasmático. Existem 6 potenciais sítios de glicosilação, sendo 3 sítios no domínio catalítico, 2 no domínio tipo-disintegrina e 1 no

domínio rico em cisteína. Na forma zimogênio a massa molecular dessa metaloprotease é 130 kDa e na forma madura é 95 kDa.

O domínio tipo-disintegrina apresenta um motivo ECD de ligação à integrina, sendo que a única integrina já descrita como ligante da ADAM17 é a integrina α5β1 (BAX *et al.*, 2004).

O domínio citoplasmático da ADAM17 apresenta 131 resíduos de aminoácidos e é rico em prolinas, o que lhe confere maior habilidade de ligação à proteínas (via os motivos SH3, resíduos 731-738 e 741-748). Seu papel já foi associado ao trânsito intracelular da ADAM17 (SOOND *et al.*, 2005). Além disso, acredita-se que o domínio citoplasmático da ADAM17 possa sofrer proteólise do tipo RIP (*regulated intramembrane proteolysis*) e atuar como fator de regulação da transcrição gênica, assim como ocorre com o domínio citoplasmático da ADAM10 (TOUSSEYN *et al.*, 2009).

Dentre os membros da família, a ADAM17 apresenta maior similaridade com a ADAM10, compartilhando até mesmo alguns substratos, como Notch, APP, CD30, CD44, CX3CL1, entre outros (EDWARDS *et al.*, 2008), mas interessantemente a similaridade entre as sequências é menor que 30%.

As vias de sinalização e o ambiente celular podem ser regulados pela atividade da ADAM17, dependendo do contexto em que o substrato e/ou receptor são clivados. GÖOZ *et al.* (2006) mostraram que a ADAM17 cliva o fator de crescimento epidermal ligado à heparina (HB-EGF), que se liga ao receptor do fator de crescimento epidermal (EGFR) e

desencadeia o processo de proliferação. Por outro lado, a ADAM17 também participa da clivagem do receptor MCSF (*macrophage colony-stimulating factor*) bloqueando o início da cascata de sinalização para a diferenciação e proliferação dos fagócitos mononucleares (ROVIDA *et al*., 2001). Outro exemplo clássico que envolve ADAM17 ou ADAM10 é a clivagem de Notch. Após a clivagem, há liberação da porção intramembrana, que sofre clivagem do tipo RIP, é transportada para o núcleo e atua como um fator de regulação da transcrição gênica. Além desses papéis, a ADAM17 também participa dos processos de comunicação entre receptores, como a transativação do EGFR via receptores associados à proteína G (GPCRs) e seus agonistas (GOOZ, 2010) (FIGURA 5).

Experimentalmente, o forbol éster PMA (*phorbol 12-myristate 13-acetate*), tem sido uma droga amplamente utilizada em estudos de ativação da ADAM17 (REDDY *et al*., 2000; DÍAZ-RODRIGUEZ *et al.*, 2002; FAN *et al.*, 2003; XU & DERYNCK, 2010) e outras proteínas transmembrana. Trata-se de um mimético do diacilglicerol, que culmina na ativação de proteína quinase C (PKC), desencadeando uma ativação bastante forte e pleiotrópica (FIGURA 3 e 4). Esse acionamento, apesar de não ser específico, promove a ativação da ADAM17.

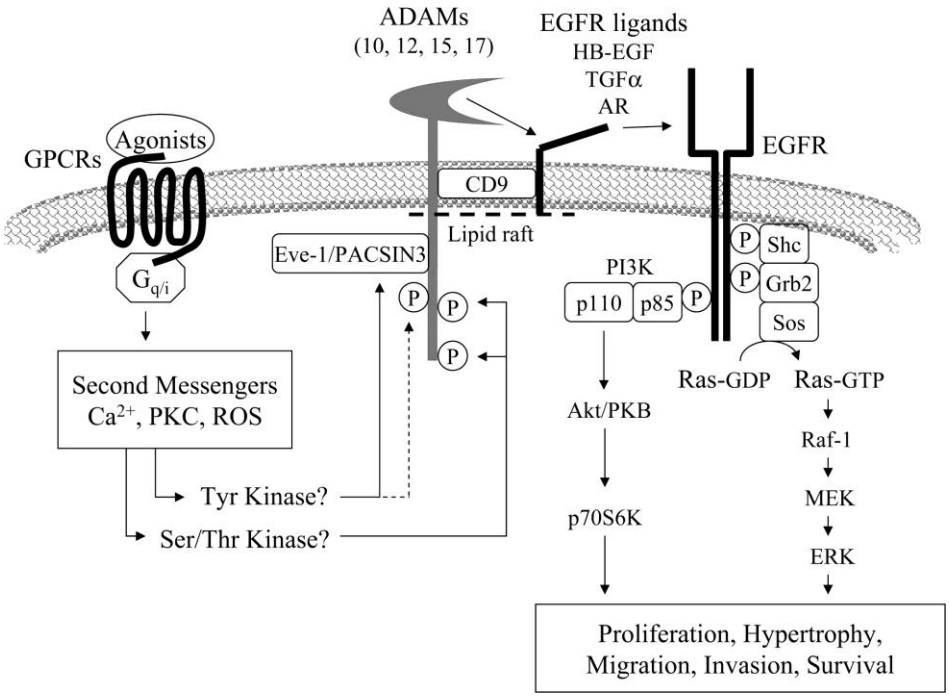


**FIGURA 4: Mecanismo de transativação do EGFR com a participação de GPCRs e ADAMs.**

Retirado de OHTSU *et al.*, 2006.

Assim como as demais ADAMs, a ADAM17 também é regulada por alguns mecanismos gerais, como a remoção do pró-domínio (LI *et al.*, 2009) durante sua passagem pela rede *trans*-Golgi (SCHLONDORFF *et al.*, 2000). É importante ressaltar que o inibidor natural da ADAM17 é o inibidor de metaloproteases TIMP3 (AMOUR *et al.*, 1998).

Outro mecanismo regulatório já descrito é o processo denominado interruptor de cisteína (*cysteine switch*), que consiste na modulação da C184 que coordena o grupo $Zn^{2+}$, impedindo que esse íon metálico se ligue ao domínio catalítico e ative a enzima. Porém, esse mecanismo parece não ser essencial para a secreção da ADAM17 na sua forma inativa, pois mutantes C184A permanecem inativos somente pela presença do pró-domínio (LEONARD *et al.*, 2005).

Espera-se que a ADAM17 madura atue na membrana, visto que é uma proteína transmembrana e seus substratos também estão ancorados à membrana, porém ensaios de imunolocalização mostram a presença da ADAM17 madura também na região perinuclear (SCHLONDORFF *et al.*, 2000). TELLIER *et al.* (2006) demonstraram que esta protease também se encontra em microdomínios de membrana, chamados de *lipid-rafts*. Tanto a localização perinuclear, quanto à presença nos microdomínios indica que a ADAM17 pode atuar em diferentes locais na célula e, dessa maneira, o controle da localização e o endereçamento da proteína torna-se um fator fundamental para a regulação da sua atividade.

Estudos anteriores mostram que a ativação de ADAM17 por fatores de crescimento ocorre pela acionamento da via de ERK1/2 (DÍAZ-RODRÍGUEZ *et al.*, 2002; FAN *et al.*, 2003) e mais recentemente, XU & DERYNCK (2010) mostraram que a via de p38 também atua diretamente na ativação da ADAM17 pela fosforilação do seu domínio citoplasmático (T735). A via de ERK1/2 foi descrita realizando a fosforilação do resíduo de T735 em células CHO e HEK293 (DÍAZ-RODRÍGUEZ *et al.*, 2002), porém o estudo de FAN *et al.* (2003) indica que também há fosforilação da S819 e desfosforilação da S791.

Estudos usando o sistema de duplo-híbrido e co-imunoprecipitação têm mostrado a interação da ADAM17 e seus domínios com diversos ligantes, como: MAD2, via domínio citoplasmático (NELSON *et al.*, 1999); SAP9, via C-terminal (SURENA *et al.*, 2009); Eve-1 (TANAKA *et al.*, 2004) e FHL2, via domínio citoplasmático (CANAULT *et al.*, 2006). Os estudos para descoberta de novos parceiros de interação da ADAM17 são fundamentais, pois trazem o conhecimento sobre como esses ligantes atuam e como ocorrem essas interações possibilitando a modulação da atividade da ADAM17 via seus interactores.

Recentemente, DANG *et al*. (2013) demonstraram que a regulação da ADAM17 pode ser específica para cada tipo de substrato. Usando TPA (um éster de forbol) e angiotensina II, que ativam a sinalização via PKC-α, houve clivagem de determinados fatores de crescimento como o TGF-α, HB-EGF e anfiregulina, enquanto que se a ativação for via PKC-δ, houve clivagem de neuroregulina. A seleção dos substratos por regulação específica mediada pela sinalização é um evento distinto da regulação da atividade

enzimática por regulação geral e de acordo com os autores pode ser um importante mecanismo a ser explorado na terapia de algumas doenças.

Considerando estes aspectos, acredita-se que a partir do conhecimento dos ligantes, das vias de sinalização e dos mecanismos de regulação das ADAMs, essas proteases podem ser alvos atrativos para o desenho racional de fármacos que modulem a ação dos seus ligantes e sejam úteis no restabelecimento da homeostase celular.

### 1.3 Tioredoxina-1: uma tiol-isomerase envolvida no balanço redox na célula

A classe das Tioredoxinas (Trx) abrange algumas das principais proteínas responsáveis pela regulação do balanço oxidativo do ambiente celular. Elas são classificadas como oxiredutases, ou seja, são proteínas capazes de reduzir as ligações dissulfeto (S-S) das proteínas, deixando seus grupos tiol (-SH) livres. Dessa reação resulta a oxidação da própria Trx, que dimeriza através da formação de ligações dissulfeto entre as cisteínas de duas moléculas dessa proteína (ARNÉR & HOLMGREN, 2000).

Desde a década de 1970, as Trxs de bactérias e bacteriófagos T4 têm sido estudadas em termos da sua estrutura e função (HOLMGREN *et al.*, 1975; SÖDERBERG *et al.*, 1978). Em bactérias, as Trxs desempenham também a atividade de chaperonas, responsáveis pelo enovelamento das proteínas (BERNDT *et al.*, 2008). A estrutura comum às Trxs de diferentes organismos é a presença de quatro hélices-α e cinco folhas-β. O

motivo CXXC liga a segunda folha-β à segunda hélice-α, deixando a proteína bem compacta, globular e estável (FIGURA 5).

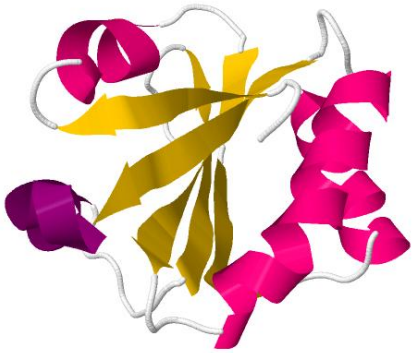

**FIGURA 5: Estrutura da Trx-1 de *E. coli.*** Três hélices- α em magenta e 1 em roxo, 5 folhas-β em amarelo. (PDB: 1xob).

As Trxs de mamíferos mais importantes e melhor descritas são a Trx-1 citoplasmática (~12 kDa) e a Trx-2 mitocondrial (~18 kDa), l (SPYROU *et al.*, 1997). Ambas apresentam o motivo CXXC, que é o sítio ativo responsável pela redução das ligações dissulfeto das proteínas-alvo. A Trx-2 apresenta uma região N-terminal de endereçamento à mitocôndria (região MTS – *mithocondrial translocation* signal). A Trx-1 além de ser encontrada preferencialmente no citoplasma é também passível de translocação para o ambiente extracelular (WORLD *et al.*, 2011), sendo que os mecanismos de

translocação não são conhecidos, porém as formas nucleares e extracelulares da Trx-1 aparecem após algum tipo de estresse oxidativo, ou seja, a forma oxidada parece ser necessária para essas translocações (ARNÉR & HOLMGREN, 2000).

Existem algumas vias pelas quais a célula é capaz de manter o ambiente intracelular reduzido e uma dessas vias canônicas é chamada de Sistema da Tioredoxina Redutase (ARNÉR, 2009). Esse sistema é composto pela Peroxirredoxina-1 (Prx-1), Trx-1, Tioredoxina Redutase (TrxR) e NADPH e é o sistema em que a Trx-1 tem seu papel melhor compreendido (FIGURA 6). MAHMOOD *et al.* (2013) incluem nesse Sistema a forma truncada da Trx (Trx-80) e o inibidor natural da Trx, chamado de TXNIP (Trx-*interating protein*).

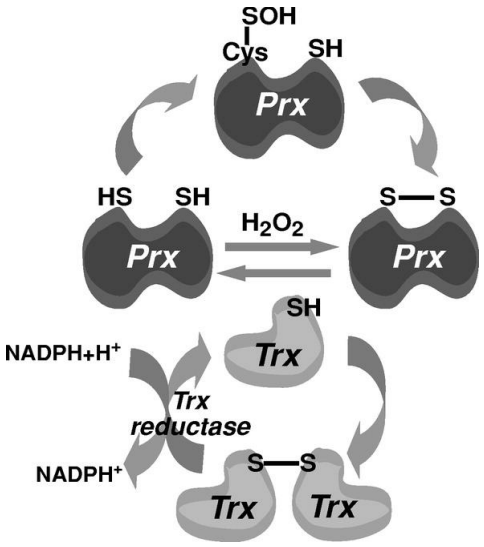


**FIGURA 6: Esquema mostrando o Sistema da Tioredoxina Redutase.** Retirado de: MARÍ *et al.*, 2010. O desacoplamento do peróxido de oxigênio (H2O2) é mediado pela Peroxiredoxina, que

após a reação fica oxidada e é regenerada pela Tioredoxina. A Tioredoxina forma um dímero pela oxidação das suas cisteínas e depois é regenerada pela ação da Tioredoxina Redutase.

Além de participar desse sistema, a Trx-1 tem sido associada à numerosas e diferentes funções, por exemplo, quando é transportada para fora da célula, adota função de sinalizadora para crescimento celular e fator quimiotáxico (WAKASUGI *et al.*, 1987; ERICSON *et al.*, 1992; MAHMOOD *et al.*, 2013), quando transportada para o núcleo é responsável por regular alguns fatores de transcrição, ligando-se ao fator nuclear *kappa* B (NF-κB) e à Proteína ativadora 1 (AP1) (SCHENK *et al.*, 1994; HIROTA *et al.*, 1997). A associação de Trx-1 com proteínas de sinalização como a quinase ASK-1 e a fosfatase PTEN (WU & CEDERBAUM, 2010; SARTELET *et al.*, 2011) também indicam que a Trx-1 atua centralmente em diferentes processos celulares, regulando suas funções e mesmo modulando cascatas de sinalização (FIGURA 7).

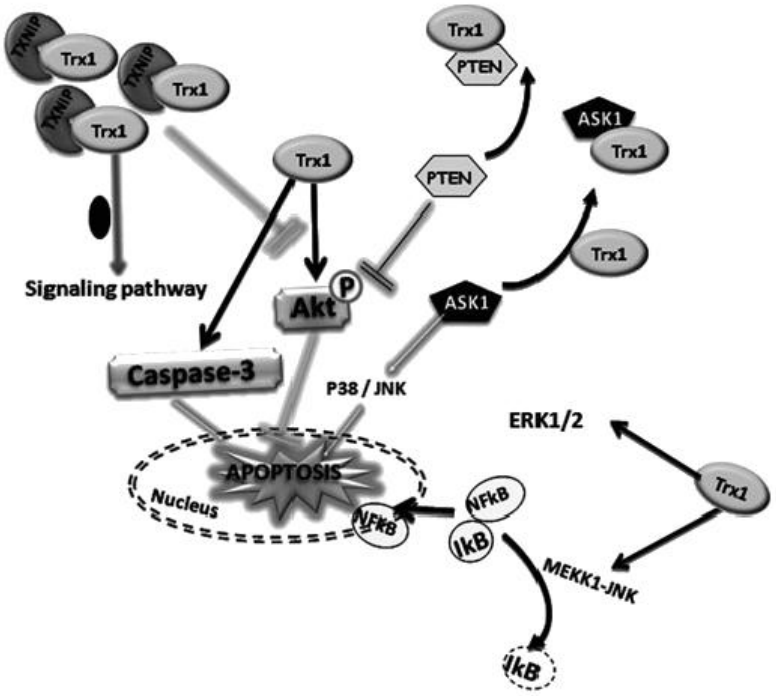


**FIGURA 7: Trx-1 uma molécula envolvida nos processos de sinalização.** Trx-1 reduzida pode inibir a apoptose através da ligação a ASK-1. Além disso, Trx-1 pode ativar a via de Akt, direta ou indiretamente, ao interagir com PTEN, inibindo sua ativação. Trx-1 também pode ativar certos componentes da via de sinalização de Junk (JNK) resultando na ativação de NF-κB. O TXNIP inibe as atividades de Trx-1. Modificado de MAHMOOD *et al.*, 2013.

# 2 OBJETIVOS

## 2.1 OBJETIVO GERAL

O objetivo geral deste trabalho foi estudar os mecanismos de ativação da ADAM17 via domínio citoplasmático pela identificação de novos ligantes.

## 2.2 OBJETIVOS ESPECÍFICOS

Os objetivos específicos foram: 1) identificar novos parceiros de interação do domínio citoplasmático de ADAM17 e 2) avaliar o papel do ligante Trx-1 na modulação da ativação da ADAM17.

# 3 RESULTADOS

## 3.1 CAPÍTULO 1

### Identification of Novel Interaction Between a Disintegrin and Metalloprotease-17 (ADAM17) and Thioredoxin-1

**Annelize Z. B. Aragão**, Maria Luiza C. Nogueira, Daniela C. Granato, Fernando M. Simabuco, Rodrigo V. Honorato, Zaira Hoffman, Sami Yokoo, Francisco R. M. Laurindo, Fabio M. Squina, Ana Carolina M. Zeri, Paulo S. L. Oliveira, Nicholas E. Sherman, Adriana F. Paes Leme

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 51, pp. 43071–43082, December 14, 2012

(doi:10.1074/jbc.M112.364513)

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## Identification of Novel Interaction between ADAM17 (a Disintegrin and Metalloprotease 17) and Thioredoxin-1*

Received for publication, April 5, 2012, and in revised form, October 24, 2012 Published, JBC Papers in Press, October 26, 2012, DOI 10.1074/jbc.M112.364513

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**Background:** The identification of potential interaction partners for TACE could be instrumental in understanding the regulation of TACE activity.
**Results:** Trx-1 interacts with the cytoplasmic domain of ADAM17.
**Conclusion:** Trx-1 regulates ADAM17 activity.
**Significance:** The data suggest a negative ADAM17 regulation in the HB-EGF shedding model.

Identification of Novel Interaction between a Disintegrin and Metalloprotease-17 (ADAM17) and Thioredoxin-1

**Annelize Z. B. Aragão[1], Maria Luiza C. Nogueira[1], Daniela C. Granato[1], Fernando M. Simabuco[1], Rodrigo V. Honorato[1], Zaira Hoffman[2], Sami Yokoo[1], Francisco R. M. Laurindo[3], Fabio M. Squina[2], Ana Carolina M. Zeri[1], Paulo S. L. Oliveira[1], Nicholas E. Sherman[4], Adriana F. Paes Leme[1]**

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*Running title**:*** *Thioredoxin-1 is a novel interaction partner of ADAM17*

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**Keywords:** ADAM17; thioredoxin-1; mass spectrometry.

Background: **The identification of potential interaction partners for TACE could be instrumental in understanding the regulation of TACE activity.**

Results: **Trx-1 interacts with cytoplasmic domain of ADAM17.**

Conclusion: **Trx-1 regulates ADAM17 activity.**

Significance: **The data suggest a negative ADAM17 regulation in HB-EGF shedding model.**

## SUMMARY

ADAM17, which is also known as TNFα-converting enzyme (TACE), is the major sheddase for the EGF receptor ligands and is considered to be one of the main proteases responsible for the ectodomain shedding of surface proteins. How a membrane-anchored proteinase with an extracellular catalytic domain can be activated by inside-out regulation is not completely understood. We characterized thioredoxin-1 (Trx-1) as a partner of the ADAM17 cytoplasmic domain that could be involved in the regulation of ADAM17 activity. We induced the overexpression of the ADAM17 cytoplasmic domain in HEK293 cells, and ligands able to bind this

domain were identified by MS after protein immunoprecipitation (IP). Trx-1 was also validated as a ligand of the ADAM17 cytoplasmic domain and full-length ADAM17 recombinant proteins by immunoblotting, immunolocalization and by solid-phase binding assay. In addition, using nuclear magnetic resonance, it was shown *in vitro* that the titration of the ADAM17 cytoplasmic domain promotes changes in the conformation of Trx-1. The MS analysis of the cross-linked complexes showed cross-linking between the two proteins by lysine residues. To further evaluate the functional role of Trx-1, we used a HB-EGF shedding cell model and observed that the overexpression of Trx-1 in HEK293 cells could decrease the activity of ADAM17, activated by either PMA or EGF. This study identifies Trx-1 as a novel interaction partner of the ADAM17 cytoplasmic domain and suggests that Trx-1 is a potential candidate that could be involved in ADAM17 activity regulation.

## INTRODUCTION

ADAMs (*A* *D*isintegrin *A*nd *M*etalloproteinase) comprise a family of membrane-associated metalloproteinases with a complex multi-domain structure composed of the following domains: metalloproteinase, disintegrin-like, cysteine-rich, epidermal growth factor-like, transmembrane and cytoplasmic domains. ADAM17, also known as the TNFα-converting enzyme (TACE), is responsible for cleaving of several growth factors, cytokines and cell surface receptors (1).

Progress has been made to identify the signaling molecules that participate in the induction of ADAM17 shedding. Several reports have questioned whether the cytoplasmic domain is involved (2) or not involved (3) in ADAM17 activation, and some mechanisms have been proposed, such as the following: 1) phosphorylation of its cytoplasmic domain at $Thr^{735}$ by p38α MAP kinase (2); 2) EGFR activation by G-protein coupled receptors involving the Src-dependent phosphorylation of ADAM17 (4) and 3) Erk MAP kinase signaling (5, 6). However, Le Gall et al. (3) showed that ADAM17 activity does not depend on intracellular signaling through the ADAM17 cytoplasmic tail, suggesting that the regulation may occur through the transmembrane domain. Additionally, it has been shown that the down-regulation of thiol isomerases enhanced ADAM17 activity by inducing changes in the redox environment and thus, a protein disulfide isomerase could be a specific regulator (7). In this context, Zhang et al. (8) suggested that $H_2O_2$ can activate ADAM17 through oxidative attack of a pro-domain thiol group and therefore lead to the disruption of its inhibitory coordination with the $Zn^{++}$ in the catalytic domain. In addition, many authors have shown the involvement of reactive oxygen species (ROS) in ADAM17 activation by p38 MAP kinase (9, 10).

Several proteins have been identified as ADAM17 cytoplasmic domain interaction partners, such as MAD2 (11), PTPH1 (12), Erk1/2 (6), FHL2 (13) and p38α MAPK (2). The cytoplasmic domain of ADAM12 has also been described as a partner of c-Src/Yes (14, 15), Grb2 (15), PI3-K (16), α-actinin-1 (17), Tks5/FISK (18), PACSIN3 (19), Eve-1 (20) and PKCε (21). However, most of these partners are not necessarily related to the proteolytic activation.

To correlate the activation of ADAM17 by inside-out regulation, we used multiple strategies to identify ADAM17 cytoplasmic domain partners and cell-based assays to analyze the functional role of the partner in ADAM17 activation. In the present study, we have demonstrated the following: 1) Trx-1 is an ADAM17 cytoplasmic domain ligand in HEK293 cells; 2) Trx-1 co-localized with the ADAM17 cytoplasmic domain and full-length ADAM17 recombinant proteins; 3) Trx-1 directly interacts with the ADAM17 cytoplasmic domain, 4) the overexpression of Trx-1 recombinant protein in the presence of PMA, which generates reactive oxygen species (ROS), decreases ADAM17 activity and finally, 5) the physiological shedding of ADAM17 substrate, such as EGF, is modulated by interaction with Trx-1. These results suggest that Trx-1 is a direct interaction partner of the ADAM17 cytoplasmic domain and could be involved in the modulation of ADAM17 activity.

## EXPERIMENTAL PROCEDURES

*Cell culture:*

HEK293 (Human Embryonic Kidney), HeLa (cervical cancer cells) and SV40-transformed mEF cells (mouse embryonic fibroblast) were cultured in DMEM with 10% FBS and supplemented with antibiotics at 37°C in 5% $CO_2$ atmosphere. The wild-type (wt) mEF cells and *Adam17-/-* mEF cells were kindly provided by Dr. Carl Blobel from Weill Medical College of Cornell University (22).

*Constructions:*

Human ADAM17 cytoplasmic domain and human Trx-1 were cloned from a cDNA library, generated from HEK293 cells. Vectors, primers sequences, insert restriction sites and cloned residues are described in the Table 1. Full-length HA-tagged ADAM17 was kindly supplied by Dr. Axel Ullrich (Department of Molecular Biology, Max-Planck Institute of Biochemistry) (23).

Plasmid encoding HB-EGF-AP, a chimeric protein used for Alkaline Phosphatase (AP) reporter assay, was kindly provided by Dr. Michael R. Freeman (Department of Surgery, Harvard Medical School, Boston) (24-26).

**TABLE 1**
**Vectors, cloned residues, and primer sequences used for ADAM17 cytoplasmic domain and Trx-1 constructions**

| Construction | Residues | Vector | Primer sequences |
|---|---|---|---|
| FLAG-ADAM17cyto | 693–824 | pcDNA3 | 5′-AAGAATTCATGGACTACAAAGACGATGACGACAAG-3′ (EcoRI)<br>5′-AATCTAGATTAGCACTCTGTTTCTTTGC-3′ (XbaI) |
| His-FLAG-ADAM17cyto | 693–824 | pET 28a (+) | 5′-TATATGCTAGCATGGACTACAAAGACGATGACG-3′ (NheI)<br>5′-ATATGGATCCTTAGCACTCTGTTTCTTTGCTG-3′ (BamHI) |
| Trx-1-HA | 1–105 | pcDNA3 | 5′-AAGGATCCATGGTGAAGCAGATCGAGAGC-3′ (BamHI)<br>5′-GGTCTAGAGACTAATTCATTAATGGTGGCTTC-3′ (XbaI) |
| His-Trx-1-HA | 1–105 | pET-28a (+)[a] | |

[a] Subcloned from pcDNA3 Trx-1-HA between BamHI and NotI.

The negative control for Immunoprecipitation (IP) experiments was a FLAG-tagged GFP, named FLAG-GFP, cloned into a pcDNA3 vector (Invitrogen) or the empty pcDNA3.

*Transient transfections:*

HEK293 and HeLa cells were transiently transfected with vectors encoding FLAG-ADAM17cyto, ADAM17-HA and FLAG-GFP using the LipofectAMINE and Plus reagent (Invitrogen), following manufacturer's

instructions. Protein overexpression was performed during 48 h for IP and confocal co-localization experiments. Trx-1-HA was also co-transfected in HEK293 cells stable expressing HB-EGF-AP using Polyethylenimine (PEI, Polysciences Inc.) during 48 h.

Wt and *Adam17-/-* mEF cells were transiently transfected with vector encoding HB-EGF-AP as described before.

*Stable transfections:*

For alkaline phosphatase reporter assay, HEK293 cells were transfected using PEI (Polysciences Inc.) and the stable transfected cells (mix population) were selected by G418 (Gibco) at 500 µg/mL for at least 2 weeks.

*Immunoprecipitation experiments:*

In order to identify ADAM17 cytoplasmic binding partners, HEK293 cells were seeded in five 150 mm dishes (Corning) until 50-60% confluence and after transient transfections with FLAG-ADAM17cyto, the cells were washed with PBS, centrifuged for 5 min at 150 × g and ressuspended in 10 mL of lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with phosphatase inhibitors (1 mM $Na_3VO_4$, 10 mM $Na_4O_7P_2.10H_2O$, 1 mM NaF, 1 mM $C_3H_7Na_2O_6P.H_2O$, Sigma). After 30 min of incubation on ice, cell lysates were centrifuged for 10 min at 10,000 × g at 4°C to clear cell debris. Cell lysates were incubated at 4°C overnight under gentle agitation with 150 µL of anti-FLAG M2 Affinity Gel (Sigma). Beads were washed three times with TBS and the immunocomplexes were eluted with FLAG peptide (Sigma) at a final concentration of 150 ng/µL, during 2 h at 4°C, under agitation. The experiment was performed three times and the samples were analyzed by LC-MS/MS.

To confirm the interaction between Trx-1 and full-length ADAM17-HA, HEK293 cells were transfected and lysed using the following lysis buffer (250 mM NaCl, 50 mM Tris, pH 8.0, containing 5 mM EDTA, 0.5% Igepal and protease inhibitors (complete mini-EDTA free, Roche). For the resin preparation, 100 µL of protein G sepharose (GE Healthcare) and 2 µg of anti-HA antibody was added to 1 mL of lysis buffer and incubated during 1 h at room temperature under gentle agitation. The resin was spun down at 12,000 × g and 300 µg of protein extract was added to the pellet and incubated for 2 h at 4°C under gentle agitation. The resin was then washed 6 times with lysis buffer and the proteins were eluted by adding Laemmli sample buffer. The interaction of ADAM-17-HA and endogenous Trx-1 were analyzed by immunoblotting.

To evaluate whether the endogenous Trx-1 interaction is dependent or independent of phosphorylation, HEK293 cells were transiently transfected with FLAG-ADAM17cyto as described above. After 48 h, the cells were washed three times with PBS (phosphate-buffered saline) and treated with PMA (50 ng/mL) or DMSO for 1 h. The proteins were immunoprecipitated and analyzed by LC-MS/MS. The data analyses were performed using QualBrowser software (Thermo Xcalibur v.2.1.), which calculates the area under the curve of extracted ion chromatogram (XIC) of peptides in a

narrow m/z range corresponding to peptides from potential interaction partners and/or phosphorylation sites.

*Analysis of ADAM17 cytoplasmic domain partners by LC-MS/MS:*

The immunocomplexes were reduced (5 mM dithiotreitol, 25 min at 56°C), alkylated (14 mM iodoacetamide, 30 min at room temperature in the dark), and digested with trypsin (Promega). The samples were dried in a vacuum concentrator and reconstituted in 20 µL of 0.1% formic acid. 4.5 µL of the resulting peptide mixture was analyzed on an ETD enabled LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled with LC-MS/MS by an EASY-nLC system (Proxeon Biosystem) through a Proxeon nanoelectrospray ion source. Peptides were separated by a 2-90% acetonitrile gradient in 0.1% formic acid using a pre-column EASY-Column (2 cm x ID100 µm, 5 µm particle size) and an analytical column PicoFrit Column (20 cm x ID75 µm, 5 µm particle size, New objective) at a flow rate of 300 nL/min over 45 min. The nanoelectrospray voltage was set to 2.5 kV and the source temperature was 200°C. All instrument methods for the LTQ Velos Orbitrap were set up in the data dependent acquisition mode. The full scan MS spectra (m/z 300-2,000) were acquired in the Orbitrap analyzer after accumulation to a target value of $1e^6$. Resolution in the Orbitrap was set to r= 60,000 and the 20 most intense peptide ions with charge states ≥ 2 were sequentially isolated to a target value of 5,000 and fragmented in the linear ion trap by low-energy CID (normalized collision energy of 35%). The signal threshold for triggering an MS/MS event was set to 1,000 counts. Dynamic exclusion was enabled with an exclusion size list of 500, exclusion duration of 60 s, and repeat count of 1. An activation q=0.25 and activation time of 10 ms were used.

Peak lists (msf) were generated from the raw data files using Proteome Discoverer version 1.3 (Thermo Fisher Scientific) with Sequest search engine and searched against Human International Protein Database (IPI) v. 3.86 (91,522 sequences; 36,630,302 residues, release July 2011) with carbamidomethylation (+57.021 Da) as fixed modification, oxidation of methionine (+15.995 Da), phosphorylation of serine, threonine and tyrosine (+79.966 Da) as variable modifications, one trypsin missed cleavage and a tolerance of 10 ppm for precursor and 1 Da for fragment ions, filtered using xcorr cutoffs (+1>1.8, +2>2.2, +3>2.5 and +4>3.5). Potential phosphorylation sites passing the scoring thresholds were manually validated.

*Immunoblotting:*

For detection of ADAM17 recombinant protein and to validate partners from immunocomplexes, samples (20 µL) were heated to 95 °C for 5 min and separated by 15% SDS-PAGE. Proteins were transferred onto nitrocellulose membrane (GE Healthcare) by semi-dry system (Bio-Rad). The nitrocellulose membrane was blocked with 5% skim milk for 2 h, and incubated with anti-FLAG (1:5000, Sigma), anti-HA (1:2000, Sigma) and anti-Trx-1 (1:1000, Abfrontier) specific antibodies for 2 h. The membranes were washed three times, each for 5 min, with 10 mL Tris-buffered saline containing 0.05%

Tween 20, and then reacted to horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, Santa Cruz Biotechnology) or mouse anti-goat IgG (1:5000, Santa Cruz Biotechnology) for 2 h. After three washes, as described above, the visualization of ADAM17 recombinant protein and Trx-1 were achieved by chemiluminescence with the ECL kit (Amersham Biosciences).

*Enrichment of membrane proteins:*

To access the expression of ADAM17 zymogen (pro ADAM17) and its mature form, we performed the membrane protein enrichment, as described by Butler *et al* (27), in HEK293 cells after Trx-1-HA overexpression followed by PMA treatment. The membrane proteins were separated by a 10% SDS-PAGE, and after it were transferred to nitrocellulose membrane and incubated with rabbit anti-TACE (1:2000, Millipore) antibody as described before.

*Confocal microscopy:*

HeLa cells were cultivated in 24-well plates containing 13-mm microscope cover glasses. After transient transfections with FLAG-ADAM17cyto, ADAM17-HA and Trx-1-HA, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, washed again and permeabilized with 0.5% Triton X-100 for 10 min. The cells were blocked with blocking solution (PBS containing 0.2% Triton and 3% non-fat dry milk) for 30 min and then incubated with primary antibodies diluted in blocking solution for 1 h. Anti-FLAG antibody (Sigma) was diluted to 1:200, anti-HA (Sigma) was diluted to 1:100 and anti-Trx-1 (Ab Frontier) was diluted to 1:100. Cells were washed with PBS and incubated with Alexa Fluor 488 or 568-conjugated antibodies (Invitrogen) diluted in blocking solution for 1 h. Finally, cells were washed with PBS, incubated with DAPI solution (4',6-diamidino-2-phenylindole) for 10 min, washed again and analyzed in a confocal microscope (Zeiss, LSM510).

*Expression and purification of recombinant His-tagged proteins:*

His-Trx-1-HA protein was expressed in BL21 (DE3) cells at 37 °C for 4 h after induction with 0.5 mM IPTG in LB or M9 medium supplemented with 1 g/L 15N-ammonium-chloride (Cambridge Isotopes, Inc) (28), for cross-linking and NMR experiments, respectively. The harvested cells were resuspended in lysis buffer (20 mM sodium phosphate, pH 6.5 containing 80 mM NaCl and 1 mM PMSF) and disrupted by lysozyme treatment (100 μg/mL, 30 min, on ice), followed by sonication (Vibracell VCX 500, Sonics & Materials, Inc.). The suspensions were centrifuged at 20,000 × g for 10 min at 4°C. The supernatant was loaded onto nickel-charged 5-mL His Trap Chelating columns (GE Healthcare) using a flow rate of 1 mL/min in buffer A (20 mM sodium phosphate, pH 6.5 containing 80 mM NaCl). Proteins were eluted using a linear gradient of 0-1 M imidazole. Affinity chromatography fractions containing His-Trx-1-HA recombinant protein were concentrated using a 10 kDa cut-off Amicon filter (Millipore), and submitted to size-exclusion chromatography using a Superdex 200 16/60 column (GE Healthcare) and flow rate of 1 mL/min in buffer A. All chromatographic steps were performed using an ÄKTA FPLC system (GE Healthcare). The purified fractions

were separated by 12% SDS-PAGE under denaturing conditions. Final protein concentration was determined by BCA protein Assay Kit (Thermo Fisher Scientific Inc.).

His-FLAG-ADAM17cyto protein was expressed in BL21 (DE3) cells at 37°C for 4 h after induction with 0.5 mM IPTG in LB medium. The cells were resuspended and lysed using the same protocol used for His-Trx-1-HA expression. His-FLAG-ADAM17cyto protein purification was the same as used for the purification of His-Trx-1-HA, except that one intermediate step in a 1-mL MonoQ ion exchange column (GE Healthcare) was necessary between the affinity and size exclusion chromatography. This ion exchange chromatography was carried on using a flow rate of 1 mL/min in buffer A (20 mM Tris-HCl, pH 8.8 containing 20 mM NaCl). The proteins were eluted using a linear gradient of 0-1M NaCl. The protein concentration was determined as described before.

*Solid-phase binding assay:*

One µg of purified His-FLAG-ADAM17cyto or His-Trx-1-HA were immobilized into a 96-well polystyrene High Bind microtiter plate (Corning Glass) in a 0.05 M sodium carbonate buffer, pH 9.6, with gentle agitation overnight at 4ºC, as described by Oliveira *et al*. (29). The wells were washed three times with wash solution (PBS supplemented with 0.05% Tween 20) and then blocked with PBS containing 5% non-fat dry milk for 2 h at RT. After blocking, the wells were washed again and the purified proteins were added in increasing concentration (0.5 – 16 nM), diluted in PBS supplemented with 0.05% Triton X-100. The plates were incubated for 2 h at RT, after which the wells were washed three times. We detected the protein binding using a colorimetric ELISA. We incubated the complexes for 1 h at RT with anti-Trx-1 (Ab Frontier) or anti-FLAG (Sigma) antibodies, both diluted 1:1000. The wells were washed three times and incubated with a peroxidase-linked horse anti-rabbit secondary antibody (Calbiochem) diluted 1:10000 for 1 h at RT. After incubation with secondary antibody, the wells were washed three times and developed with a buffer containing ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid, Sigma), 0.1 M citric acid and 0.03% $H_2O_2$ (Merck). The absorbance was measured at 405 nm. The experiment were performed in triplicates and repeated twice.

*Nuclear Magnetic Resonance Spectroscopy Analysis:*

Experiments that correlate the frequencies of the amide proton and the nitrogen for each amino acid in the protein can yield information about interaction sites in the protein, as well as the strength of the interaction (30). The signals that change positions in the $^{15}$N-HSQC spectra of the Trx1 protein correspond to main chain amide groups from amino acids that interacted with the unlabeled ADAM17cyto protein. NMR experiments were performed using a Varian/Agilent Inova spectrometer operating at a $^{1}$H Larmor frequency of 599,887 MHz and temperature of 25°C. For these experiments, the spectrometer was equipped with a triple resonance cryogenic probe and a Z pulse-field gradient unit. The $^{15}$N labeled Trx-1 sample was dissolved in 20 mM phosphate buffer, pH 6.5,

containing 80 mM NaCl and 5% (v/v) $D_2O$, at a final concentration of 0.7 mM. Water suppression was achieved by low-power continuous wave irradiation over the relaxation delay or using the WATERGATE method. All data were processed using NMRPipe and NMRVIEW software packages (31, 32). Prior to Fourier transformation, the time domain data were zero-filled in all dimensions. When necessary, a fifth order polynomial baseline correction was applied after transformation and phasing. Experiments were performed in one and two dimensions (2D), with double resonance techniques to correlate $^{1}H$ and $^{15}N$ from the main chain amino acids (15N-HSQC) (33). The hydrogen proton 1D spectrum was acquired in order to verify that the protein was structured, and 2D experiments (15N-HSQC) (32) were performed to identify interactions between the proteins His-Trx-1-HA and His-FLAG-ADAM17cyto. For this, a His-Trx-1-HA protein sample was labeled with $^{15}N$ and the His-FLAG-ADAM17cyto protein expressed in unlabeled media (LB) and they were titrated in different proportions (50:1, 25:1, 10:1, 5:1, 1:1 and 2:1, respectively).

*Cross-linking analysis between His-FLAG-ADAM17cyto and His-Trx-1-HA by mass spectrometry:*

Cross-linking reactions were performed by incubating the $5 \times 10^{-10}$ mol of each purified cytoplasmic domain and purified Trx-1 recombinant proteins with 1.25 mM DSS (disuccinimidyl-suberate, spacer arm length: 11.4 Å, Sigma-Aldrich) for 2 h at room temperature, followed by quenching with Laemmli sample buffer. DSS-cross-linked cytoplasmic domain–Trx-1 complexes were identified as shifted bands in 12% SDS-PAGE. The protein complexes of each band were digested with trypsin as described before. The samples were dried in a vacuum concentrator and reconstituted in 100 µL of 0.1% formic acid. 4.5 µL of the resulting peptide mixture was analyzed in LTQ Velos Orbitrap. The MS analysis were performed as described before, except for the instrument methods in LTQ Velos Orbitrap were set up in the data dependent acquisition mode of HCD fragmentation. The resolution in the Orbitrap system was set to r= 60,000 and the 5 most intense peptide ions with charge states $\geq 2$ were sequentially isolated to a target value of 50,000 and fragmented in HCD with normalized collision energy of 40% with the resolution in the Orbitrap system was set to r= 7,500 for MS/MS. The signal threshold for triggering an MS/MS event was set to 80,000 counts and activation time of 0.1 ms was used. Dynamic exclusion was enabled with exclusion size list of 400 and exclusion duration of 60 s, and repeat count of 2. For protein identification, peak lists (msf) were generated from the raw data files using Proteome Discoverer version 1.3 (Thermo Fisher Scientific) with Sequest search engine and searched against Human International Protein Database (IPI) v. 3.86 (91,522 sequences; 36,630,302 residues, release July 2011) with carbamidomethylation (+57.021 Da) as fixed modification, oxidation of methionine (+15.995 Da) and chemical cross-linked with disuccinimidyl suberate-DSS (mass of dead-end cross-linking: 156.07864 Da) as variable modifications, one trypsin missed cleavage and a tolerance of 10 ppm for precursor and 0.02 Da for fragment ions.

For cross-linked analysis, the raw data files generated by Xcalibur v.2.1. (Thermo Fisher Scientific) were converted to a peak list format (mgf) using Proteome Discoverer version 1.3 (Thermo Fisher Scientific). The mgf files were analyzed in MassMatrix software (www.massmatrix.net) (34) to automatically search chemical cross-linkage against databases containing the ADAM17cyto and Trx-1 amino acid sequences, according to software instructions. The parameters for cross-linking analysis used in MassMatrix software were carbamidomethylation (+57.021 Da) as fixed modification, oxidation of methionine (+15.995 Da) as variable modifications, chemical cross-linked with disuccinimidyl suberate-DSS (138.06808 Da) non-cleavable by enzymes, four trypsin missed cleavages and a tolerance of 10 ppm for precursor and 0.02 Da for fragment ions. Search results with high confidence (MassMatrix pp score >30) and potential cross-linked peptides were manually validated for b and y ion series containing α and β chains (35, 36). This experiment was performed three times.

*"In silico" analysis:* A high quality homology model of the human Trx-1 was built using multiple templates found on the protein data bank (PDB) with high identity and 100% coverage using Yasara (37). To elucidate the interacting region, an *ab initio* model of the ADAM17 cytoplasmic domain was obtained from I-Tasser (38) and evaluated by C-Score and TM-Score (39) approaches. A customized docking algorithm was developed through Rosetta (40) using cross-link information as constraints of the search space. Resulting decoys were assayed on energy values and lysine pair distance. Low energy models were validated through an automated DSS bonding protocol generating a final theoretical model of the interaction interface. Binding energy contributions of each interface residue and protein stability were calculated using an alanine scanning to evaluate only the interface residues of ADAM17 during a 10 nanoseconds molecular dynamics simulation with explicit solvent using Yasara. To each 25 ps a snapshot is extracted and each interface residue is mutated to alanine. Then, the whole complex had its side chains optimized using both a standard rotamer library and the force field parameters. For each mutation the binding energy of Trx-1 to wild-type ADAM17 is compared to mutated ADAM17. The difference corresponded to the level of importance of the mutated residue for the interaction interface. The binding energy function used in the calculations considered, besides the interaction potential, the solvation effects given by the sum of the coulombic and van der walls energy values plus the product of the accessible surface area multiplied by the entropic cost of exposing 1 Å2 of the protein surface.

*Analysis of ADAM17 activity on AP reporter assay:*

HEK293 cells stably transfected with HB-EGF-AP were seeded into 100-mm dishes and co-transfected with transient Trx-1-HA and empty vector (negative control). After 48 h, cells were trypsinised, counted to $3 \times 10^5$ cells per well and seeded in 24 well-plate (Corning). In the following day, the cells were starved during 4 h and activated with PMA (50 ng/mL) or EGF (100 ng/mL) (41) during 1 h in a phenol-

free medium. The cleavage of HB-EGF-AP was measured after overnight incubation. Briefly, 100 µL of conditioned media were collected of each well and added to individual wells of a 96-well plate containing 100 µL of AP buffer (0.5 M Tris-HCl, pH 9.5, containing 5 mM p-nitrophenyl phosphate disodium, 1 mM diethanolamine, 50 µM $MgCl_2$, 150 mM NaCl, 5 mM EDTA) and measured at 405 nm. Three independent experiments were performed in duplicates for PMA activation and in triplicates for EGF activation.

To confirm ADAM17 is the sheddase responsible for PMA induced HB-EGF cleavage, wt and *Adam17-/-* mEF cells expressing HB-EGF-AP were activated by PMA as described above. The conditioned media was incubated with AP buffer. To assess the amount of total HB-EGF-AP expressed, the cells were collected and the lysates were also incubated with AP buffer. The AP ratio was calculated as previously described (3). Three independent experiments were performed in triplicates.

To indirectly evaluate the effect of Trx-1 overexpression on HB-EGF-AP levels, the membrane proteins of HEK293 cells co-transfected with Trx-1-HA were collected by the enrichment of membrane protein protocol (27). The proteins were separated by a 12% SDS-PAGE under non-denaturing conditions and the AP activity was assessed onto nitrocellulose membrane after incubation with BCIP/NBT (Sigma), according to the manufacturer's protocol.

*Analysis of mRNA expression levels:* In order to analyze the mRNA expression of Trx-1, ADAM17 and HB-EGF, HEK293 cells stably expressing HB-EGF-AP were co-transfected with Trx-1-HA, as described above. Total RNA was obtained using the TRIzol reagent (Invitrogen Corporation) and 3 µg of total RNA were used for retro-transcription using the First-Strand cDNA Synthesis Kit (GE Healthcare). Real-time quantitative PCR (qRT-PCR) was performed using SYBR® Green PCR Master Mix (Applied Biosystems), and the dissociation curves were performed to confirm the specificity of products. The primers sequences were: Trx-1 5'-GGACGCTGCAGGTGATAAACTTGT-3' and 5'-TGGCGTGCATTTGACTTCACACTC-3'; ADAM17 5'-GGACCCCTTCCCAAATAGCA-3' and 5'-ATGGTCCGTGAGATCCTCAAA-3'; HB-EGF 5'-AGCTCTTTCTGGCTGCAGTTCTCT-3' and 5'-ACTGTATCCACGGACCAGCTGCTA-3'. The threshold cycles (CT) values of target genes were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, and relative expression ratios were calculated by the $2^{-\Delta\Delta}$ Ct method. Three independent experiments were performed with triplicates.

*Statistical analysis:* For the statistical analysis of HEK293 AP assays, we performed one-way ANOVA followed by Tukey test. For mEF cells AP assays, we used one-way ANOVA followed by Bonferroni test to compare within each wt and *Adam17-/-* mEF cells (control *vs* PMA). The significance level was stated at 0.05 (GraphPad Prism version 5 for Windows).

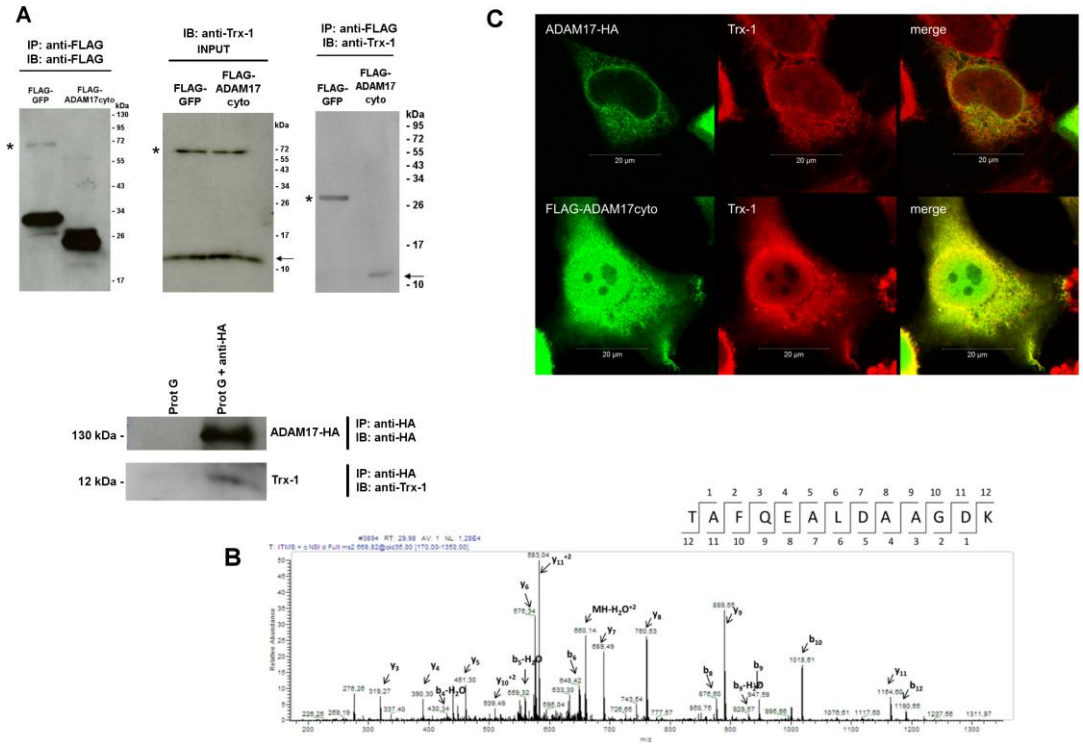


FIGURE 1. Thioredoxin-1 is revealed as novel ligand of ADAM17. HEK293 cells were transfected with FLAG-ADAM17cyto, after 48 h the intracellular proteins were collected and submitted to immunoprecipitation (IP) followed by MS and immunoblotting (IB). (A) The first of the three panels show the IB of the FLAG-tagged ADAM17 cytoplasmic domain and the negative control after IP confirming the protein expressions. Following, it shows the input of protein lysates and the detection of endogenous Trx-1 in both conditions. Finally, it confirms the Trx-1 interaction with FLAG-ADAM17cyto by IB. Arrows indicate the identification of Trx-1 bands and asterisks indicate unspecific bands. The lower panel shows the expression of full-length ADAM17-HA and its negative control by IB. Following, it also demonstrates that the endogenous Trx-1 interacts with full-length ADAM17-HA by IP followed by IB. (B) Manual validation of Trx-1 peptide, spectra containing b and y ion series. (C) Confocal immunofluorescence confirmed the co-localization between endogenous Trx-1 and full-length ADAM17-HA (upper panel) and likely endogenous Trx-1 and FLAG-ADAM17cyto (lower panel) in HeLa cells.

## RESULTS

*Identification of thioredoxin-1 as ADAM17 cytoplasmic domain interaction partner by LC-MS/MS* – To identify the interaction partners of the intracellular ADAM17 domain, the recombinant protein was expressed in HEK293 cells (Figure 1A, left panel). After immunoprecipitation (IP), the complexes were eluted with FLAG peptide, digested with trypsin and analyzed by LC-MS/MS. Three experiments were performed with a negative control (FLAG-GFP recombinant protein). Among the

proteins found, we identified Trx-1 only in the IP with FLAG-ADAM17cyto compared with the negative control. One peptide of Trx-1 was manually validated (Figure 1B).

*Validation of thioredoxin-1 as a cytoplasmic domain partner by immunoblotting* – In order to validate the interaction between FLAG-ADAM17cyto and Trx-1, an aliquot of immunoprecipitation complexes with FLAG-ADAM17cyto and negative control (FLAG-GFP recombinant protein) were separated by SDS-PAGE and analyzed by immunoblotting. This result showed the presence of endogenous Trx-1 only in the IP with FLAG-ADAM17cyto (Figure 1A, right panel). Furthermore, in the IP using full-length ADAM17-HA, Trx-1 was also identified by immunoblotting (Figure 1A, lower panel).

*Thioredoxin-1 co-localizes with cytoplasmic domain and full-length ADAM17 recombinant proteins in HeLa cells* – After the validation of the interaction between Trx-1 and FLAG-ADAM17cyto, we evaluated whether the proteins co-localize. The recombinant proteins, FLAG-ADAM17cyto and full-length ADAM17-HA, were transiently expressed in HeLa cells and confocal immunofluorescence analysis showed that both proteins were co-localized with endogenous Trx-1 (Figure 1C).

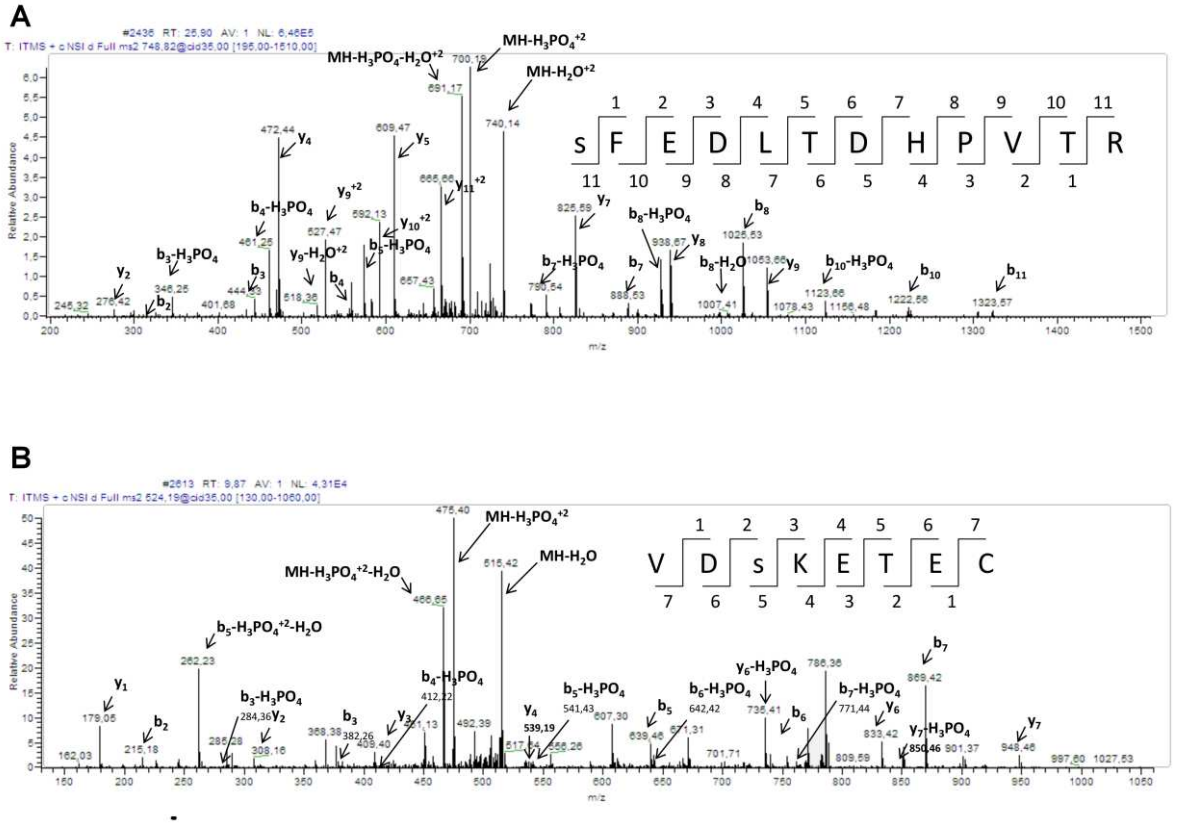


FIGURE 2. Manual validation of phosphorylation sites at ADAM17 cytoplasmic domain. MS

analyses showed the phosphorylation of ADAM17 cytoplasmic residues: $S^{791}$ and $S^{819}$. Spectra containing b and y ion series were manually annotated for validation of $pS^{791}$ (A) and $pS^{819}$ (B).

*Thioredoxin-1 interaction is independent of the phosphorylation of ADAM17 residues: $S^{791}$ or $S^{819}$* – To evaluate whether the endogenous Trx-1 interaction is dependent or independent of phosphorylation, we overexpressed FLAG-ADAM17cyto in HEK293 cells and the cells were treated with PMA. After IP, eluted complexes were digested with trypsin and analyzed by LC-MS/MS. By XIC analysis, we observed that the PMA treatment resulted in a lower phosphorylation of $pS^{791}$ and a higher phosphorylation of $pS^{819}$ than the control cells, considering the ratio between XIC of phosphorylated peptides in the presence of PMA and DMSO was higher than 1.5-fold change (Table 2). However, Trx-1 was found in both preparations with the same abundance (Table 2). The spectrum of phosphorylated peptide was manually validated, confirming the phosphorylation sites at $pS^{791}$ and $pS^{819}$ (Figure 2).

**TABLE 2**
**Semiquantitative analysis of ADAM17 and Trx-1 peptides after PMA activation by XIC**

| Proteins (residues) | Peptide sequence | *m/z* range[a] | XIC of peptide from $Me_2SO$ treatment | XIC of peptide from PMA treatment | PMA/$Me_2SO$[b] | PMA/$Me_2SO$ normalized[c] |
|---|---|---|---|---|---|---|
| ADAM17 (739–753) | LQPAPVIPSAPAAPK | 728.9224–728.9448 | $1.42 \times 10^8$ | $1.11 \times 10^8$ | 0.78 | 1.00 |
| ADAM17-$Ser^{791}$ | SFEDLTDHPVTR | 708.8350–708.8500 | $6.54 \times 10^7$ | $8.00 \times 10^7$ | 1.22 | 1.56 |
| ADAM17-$Ser(P)^{791}$ | pSFEDLTDHPVTR | 748.8170–748.8320 | $1.99 \times 10^6$ | $3.72 \times 10^5$ | 0.18 | 0.23 |
| ADAM17-$Ser^{819}$ | VDSKETEC | 484.1860–484.1942 | $7.65 \times 10^4$ | $1.76 \times 10^5$ | 2.3 | 2.94 |
| ADAM17-$Ser(P)^{819}$ | VDpSKETEC | 524.1830–524.1950 | $1.51 \times 10^4$ | $2.39 \times 10^5$ | 15.8 | 20.2 |
| Trx-1 (9–21) | TAFQEALDAAGDK | 668.8130–668.8300 | $1.78 \times 10^5$ | $1.68 \times 10^5$ | 0.94 | 1.20 |

[a] *m/z* range selected for XIC in QualBrowser software.
[b] PMA/$Me_2SO$ means the ratio between XIC of peptide from PMA treatment and XIC of peptide from $Me_2SO$ treatment.
[c] PMA/$Me_2SO$ normalized means the ratio of each peptide was normalized by the ratio of XIC of ADAM17 (739–753) peptide, which was set at 1.00.

*ADAM17 cytoplasmic domain interacts with Trx-1 by solid-phase binding assays* – We demonstrated that the interaction between His-FLAG-ADAM17cyto and His-Trx-1-HA recombinant proteins occurs in a concentration-dependent manner by immobilizing either Trx-1 or ADAM17cyto (Figures 3A and 3B, respectively).

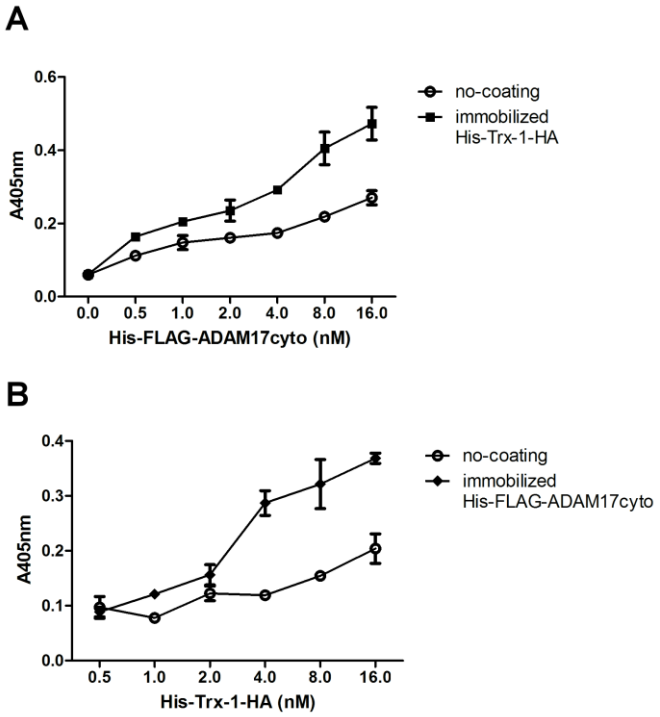


FIGURE 3. ADAM17 cytoplasmic domain and Trx-1 recombinant proteins can interact in a concentration-dependent manner. The solid-phase binding assays were performed using either Trx-1 or ADAM17cyto recombinant proteins. (A) Nanomolar concentration of His-FLAG-ADAM17cyto (0 – 16 nM) was incubated to immobilized His-Trx-1-HA. (B) Nanomolar concentrations of His-Trx-1-HA (0.5 – 16 nM) was incubated to immobilized His-FLAG-ADAM17cyto. Both incubations were performed during 2 h at RT. The negative control was the same concentrations of the recombinant proteins, but without plate coating. Two experiments were performed with triplicates.

*ADAM17 cytoplasmic domain interacts with Trx-1 by NMR* - To evaluate the interaction between Trx-1 and ADAM17cyto, the proteins were expressed in bacteria, purified by affinity chromatography and analyzed by NMR. The NH signals of the main chain protein (His-Trx-1-HA) that showed marked changes in their chemical shifts frequencies during the titration with the binding protein (His-FLAG-ADAM17cyto) were used to confirm the interaction between the proteins. These signals correspond to the labeled protein amino acids that interacted with the unlabeled protein (Figure 4).

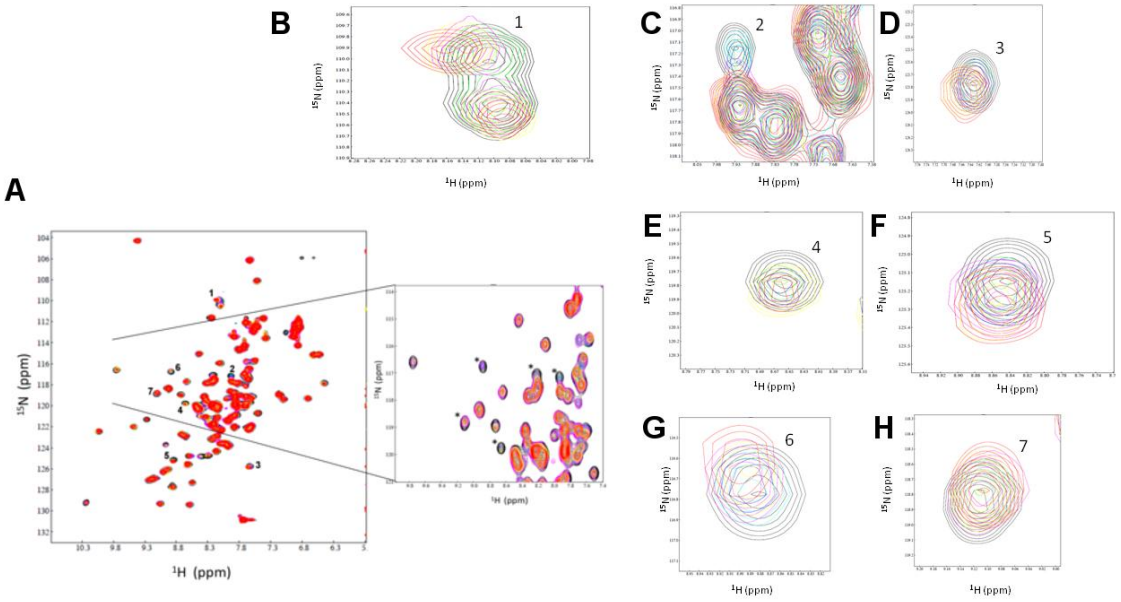


FIGURE 4. ADAM17 cytoplasmic domain and Trx-1-HA recombinant proteins showed interaction by NMR. (A) 15N-HSQC spectrum at 25°C of the Trx-1 recombinant protein (black) titrated with the ADAM17 cytoplasmic domain in the proportions 50:1 (cyan), 25:1 (blue), 10:1 (green), 5:1 (magenta), 1:1 (yellow) and 1:2 (red). Small changes in the chemical shift of some peaks were detected (inset, *) indicating an interaction between the proteins. Panels (B) to (H) represent the zoom of the peaks 1, 2, 3, 4, 5, 6 and 7 respectively.

*Chemical cross-linking coupled with mass spectrometry* - To evaluate the interaction between Trx-1 and FLAG-ADAM17cyto, we chemically cross-linked the recombinant purified proteins. Initially, the cross-linked proteins (His-FLAG-ADAM17cyto and His-Trx-1-HA) were separated by SDS-PAGE and the three main bands of 170 kDa, 55 kDa and 34 kDa were digested with trypsin. After manually verifying possible hits from MassMatrix search results, we identified one crosslink with high confidence (MassMatrix pp score >30). The 170 kDa-band showed the identification of cytoplasmic domain and Trx-1 complexes by tryptic peptides $^{726}$IIKPFPAPQTPGR$^{738}$ and $^{82}$KGQK$^{85}$, respectively, cross-linked by the side chains of the underlined lysine residues. This experiment was repeated three times and the same cross-linked peptides were confirmed. The validation of cross-linked peptides is shown in the Figure 5A.

*In silico analysis proposes a model for the interaction*– Our homology model of the human Trx-1 (Figure 5C) presented a RMSD of 1.089 Å compared to the chimeric human and *E. coli* protein (PDB 1M7T) and an overall Z-Score of 1.008, that indicated a good quality for the model. The *ab initio* model showed a C-Score of 2.59 and TM-Score of 0.41+-0.14. Our studies suggest that the ADAM17 cytoplasmic domain is very flexible and is presented here in an acceptable topology. The custom docking algorithm resulted

5,000 decoys that were filtered by binding energy and compatible cross-link lysine pair distance (Figures 5B and 5C). Best fitted model after the molecular dynamics simulation exhibited an overall mean Cα-RMSD of 3Å and further analysis demonstrated the high flexibility of the ADAM17 cytoplasmic domain with a relative mean RMSD of 5.722 Å when compared to Trx-1 (Figure 5D). Distribution of differences of binding energy suggests the contribution of each interface residue of the cytoplasmic domain of ADAM17 (Figure 5E). Residues with average above zero showed an indication that mutation disturbed the interaction surface, and thus, must be important to keep the complex in the bound configuration.

*The increase of Trx-1 expression modulates HB-EGF shedding upon activation of ADAM17 with PMA* – After the characterization of the interaction between Trx-1 and FLAG-ADAM17cyto, we evaluated the effect of overexpression of Trx-1 on ADAM17 activity. For that, HEK293 cells stably expressing HB-EGF-AP were transiently transfected with pcDNA-Trx-1-HA and treated with PMA. By analysis of AP activity in the conditioned media of the cells, HEK293 overexpressing Trx-1 showed lower HB-EGF shedding compared with control cells (Figure 6A, $p<0.05$, ANOVA followed by Tukey test). In addition, using wt and *Adam17-/-* mEF cells, we confirmed ADAM17 is the sheddase responsible for PMA induced HB-EGF cleavage (Figure 6G, $p<0.05$, ANOVA followed by Bonferroni test). The expression of Trx-1 in the same experimental conditions was confirmed by qRT-PCR (Figure 6B). Furthermore, we demonstrated that Trx-1-HA overexpression did not affect the HB-EGF-AP expression on cell surface or the mRNA levels (Figures 6C and 6D, respectively), neither alters the mRNA levels of ADAM17 (Figure 6E), nor the pro and mature forms of ADAM17 (Figure 6F).

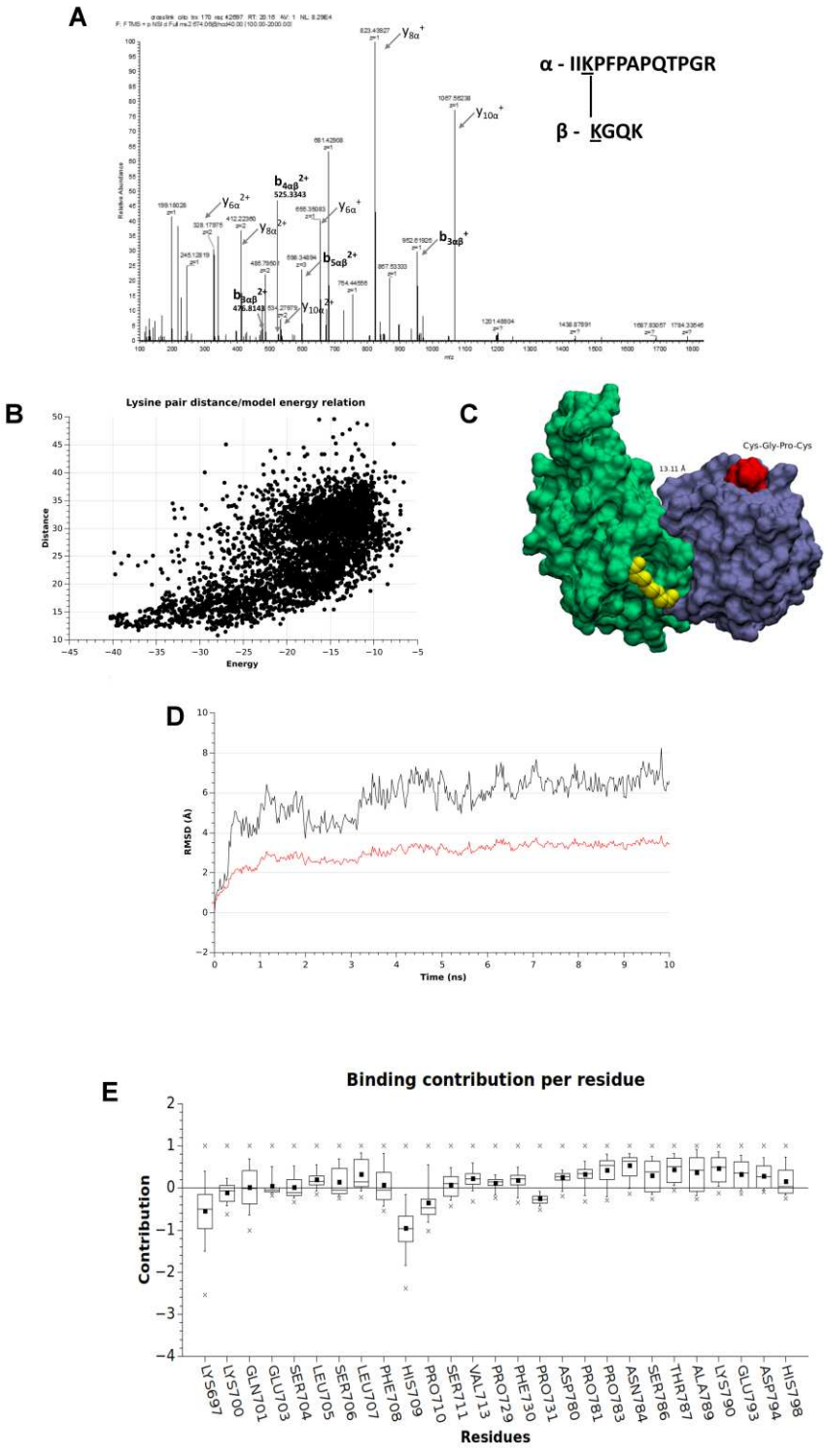
A
α - IIKPFPAPQTPGR
β - KGQK
B
Lysine pair distance/model energy relation
Distance
Energy
C
Cys-Gly-Pro-Cys
13.11 Å
D
RMSD (Å)
Time (ns)
E
Binding contribution per residue
Contribution
LYS697
LYS700
GLN701
GLU703
SER704
LEU705
SER706
LEU707
PHE708
HIS709
PRO710
SER711
VAL713
PRO729
PHE730
PRO731
ASP780
PRO781
PRO783
ASN784
SER786
THR787
ALA789
LYS790
GLU793
ASP794
HIS798
Residues

FIGURE 5. Interaction between ADAM17 cytoplasmic domain and Trx-1. (A) Purified recombinant proteins Trx-1 and ADAM17 cytoplasmic domain, were incubated, chemically cross-linked, digested with trypsin and analyzed by MS. MS/MS spectra were manually validated for b and y ion series of the α (peptide of ADAM17 cytoplasmic domain) and β (peptide of Trx-1) chains. (B) Results from the custom docking algorithm shows the relationship between low energy values and cross-link acceptable distance. (C) Best conformation based on both cross-link distance and energy value. ADAM17 cytoplasmic domain is colored in green and Trx-1 is depicted in blue. DSS is shown in van der Waals representation (yellow) and the catalytic domain of Trx-1 is show in red. (D) RMSD plot for the 10 nanoseconds simulation of the Trx-1 and ADAM17 cytoplasmic domain complex, whereas the red line represent the overall RMSD and the black line the relative RMSD between the ADAM17cyto and overall Trx-1. (E) Normalized boxplot of individual residue contributions from the ADAM17cyto through the 10 ns molecular dynamic simulations. Values above zero indicate a high contribution for the complex binding energy and should be targeted for point mutations.

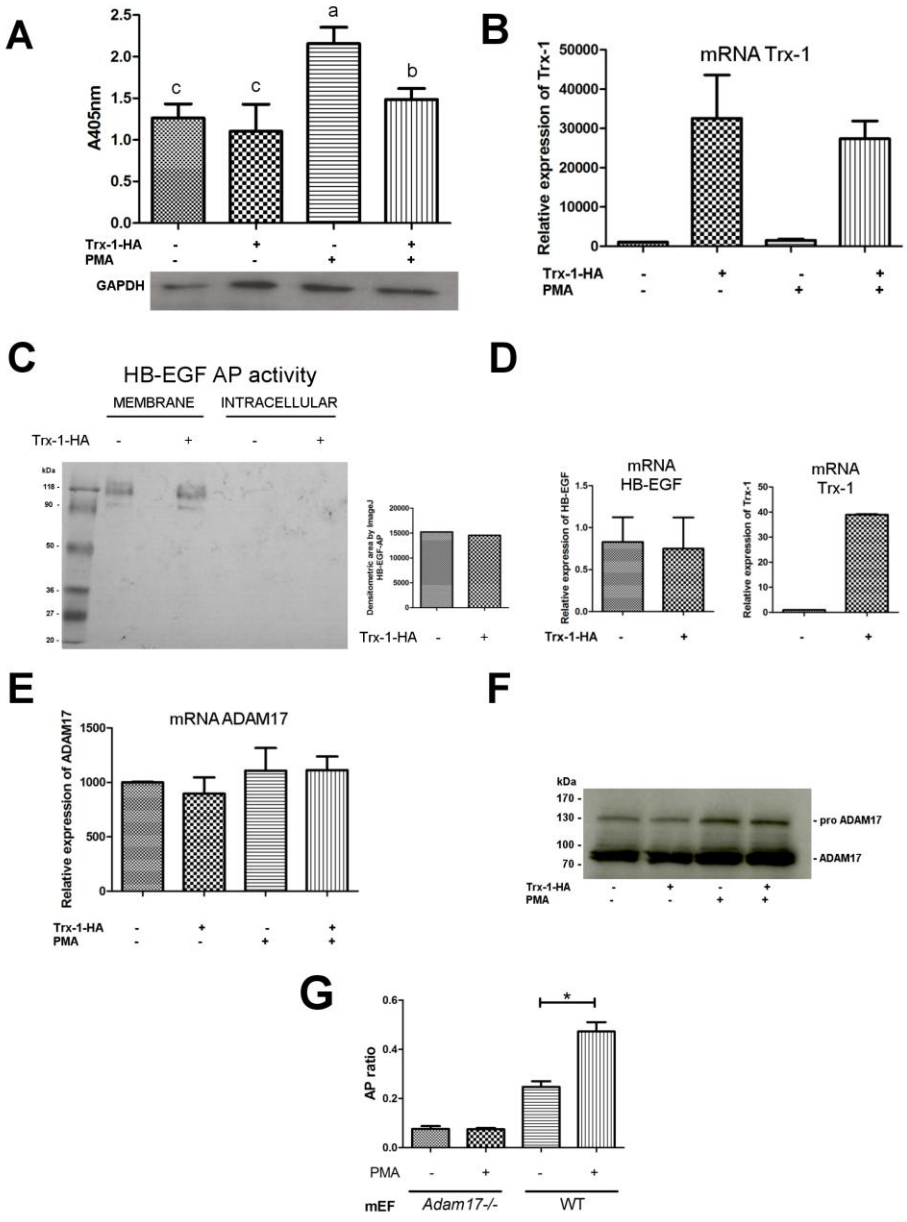


FIGURE 6. Thioredoxin-1 modulates HB-EGF shedding in HEK293 cells under PMA stimulation. HEK293 cells stably expressing HB-EGF-AP were transiently transfected with Trx-1 recombinant protein. After 48 h, cells were submitted to a starvation period of 4 h followed by PMA activation during 1 h. Supernatant was collected and evaluated by AP assay. (A) AP reporter assay indicates that Trx-1 decreases the HB-EGF shedding in HEK293 cells after PMA treatment. Three independent experiments were performed in duplicates (one-way ANOVA followed by Tukey test. Different letters indicate statistically difference at $p<0.05$). (B) qRT-PCR confirms the expression of Trx-1-HA after transient transfection in the same experimental conditions used before. (C) Alkaline phosphatase activity indirectly shows HB-EGF expression after the enrichment of

membrane proteins from HEK293 cells stably expressing HB-EGF-AP and transiently expressing Trx-1-HA. Densitometry of the alkaline phosphatase substrate bands are shown in the right panel. (D) The expressions of HB-EGF and Trx-1 in HEK293 cells are also confirmed by qRT-PCR. (E) The increase in the expression of Trx-1 do not change the ADAM17 mRNA levels and likely (F) the proADAM17 and its mature form after membrane protein enrichment in HEK293 cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (G) To confirm ADAM17 is the sheddase responsible for PMA induced HB-EGF cleavage, the wt and *Adam17-/-* mEF cells were transiently transfected with the vector encoding HB-EGF-AP and following, activated by PMA. AP ratio was calculated between the supernatant AP activity and the total AP activity in the cell lysate plus supernatant from three identically prepared wells, and averaged (3). Three independent experiments were performed in triplicates (one-way ANOVA followed by Bonferroni test for selected pairs of columns. * indicates statistical difference at $p<0.05$).

*The increase of Trx-1 expression modulates HB-EGF shedding upon activation of ADAM17 with EGF* - We demonstrated that the activation of ADAM17 with EGF is decreased in the presence of Trx-1 (Figure 7, $p<0.05$, ANOVA followed by Tukey test).

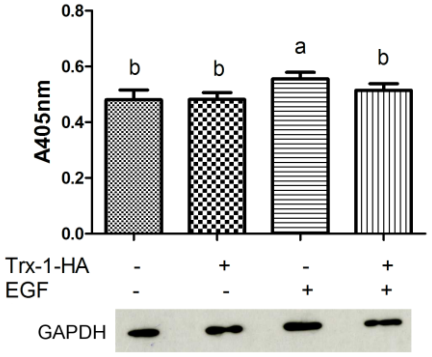


FIGURE 7. Thioredoxin-1 modulates HB-EGF shedding in HEK293 cells under physiological stimulation by EGF. HEK293 cells stably expressing HB-EGF-AP were transiently transfected with Trx-1-HA. After 48 h, cells were submitted to starvation period of 4 h followed by EGF activation during 1 h. Supernatant was collected and evaluated by AP reporter assay. The results indicate that Trx-1 negatively modulates the HB-EGF shedding under EGF stimulation. Three independent experiments were performed in triplicates (One-way ANOVA followed by Tukey test. Different letters indicate statistically difference at $p<0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

## DISCUSSION

ADAMs are one of the main proteases responsible for the ectodomain shedding of surface membrane proteins, however, how membrane-anchored proteinases can be activated by inside-out regulation remains unclear. To identify the interaction partners of the cytoplasmic domain and to correlate them with a functional role in ADAM17 activation, we induced the expression of the ADAM17 cytoplasmic domain in HEK293 cells and identified the binding partners using mass spectrometry.

We found thioredoxin-1 as a ligand of the ADAM17 cytoplasmic domain by MS and immunoblotting (Figures 1A and 1B). The immunoprecipitation data confirmed that full-length ADAM17 also interacts with Trx-1, which suggests that the interaction is not restricted to the soluble cytoplasmic recombinant protein (Figure 1A, lower panel). Co-localization assays further validated the interaction, showing that endogenous Trx-1 co-localizes with the ADAM17 cytoplasmic domain and full-length ADAM17 recombinant proteins (Figure 1C, upper and lower panels, respectively).

ADAM cytoplasmic domains are kinase targets (2, 6, 42) so, we evaluated whether the phosphorylation state could interfere in Trx-1 binding. We found that the Trx-1 interaction with the cytoplasmic domain is not dependent on $pS^{791}$ and $pS^{819}$ because changes in the phosphorylation pattern induced by PMA treatment (42), such as the decrease in $pS^{791}$ and increase in $pS^{819}$, did not change the abundance of Trx-1 binding (Table 2).

To evaluate the interaction of Trx-1 and ADAM17 cytoplasmic domain, we used three different approaches: solid-phase binding assay, NMR and chemical cross-linking coupled with MS. The solid-phase binding assays demonstrated that the interaction between the proteins occurs in a concentration-dependent manner (Figure 3A/B). From NMR studies, we also determined that the presence of the ADAM17 cytoplasmic domain promotes perturbations in the conformational dynamics of the Trx-1 (Figure 4). The MS analysis of the complexes showed the interaction between the two proteins to be cross-linked by the side chains of the lysine residues (Figure 5A) in the tryptic peptides of ADAM17 cytoplasmic domain and Trx-1, $^{726}$II<u>K</u>PFPAPQTPGR$^{738}$ and $^{82}$<u>K</u>GQK$^{85}$, respectively. As previously reported by Willems et al. (7), the interaction of thiol isomerases is rapid and not very stable in the reduced form; therefore, this approach that stabilized the complex by chemical cross-linking in vitro made it possible to confirm this interaction through the side chain of lysine residues that showed at least a 11.4 Å of distance (based on the DSS spacer arm). In addition, based on the *in silico* analysis of the protein-protein interaction between ADAM17 cytoplasmic domain and Trx-1 (Figure 5B), it can be observed that the active site residues of Trx-1 are not in the contact with the interface region of ADAM17 cytoplasmic domain and are free for binding to different substrates and to thereby perform different activities. Despite the high flexibility of ADAM17 cytoplasmic domain of ADAM17, the interface region is kept along molecular dynamic simulation. Residues that are considerably contributing to the binding in ADAM17 cytoplasmic domain are observed to be above zero (Figure 5E).

Trx-1 is known to act as a reductase via a dithiol/disulfide exchange reaction between two cysteine residues in the active site, -Cys$^{32}$-Gly-Pro-Cys$^{35}$-, on oxidized protein substrates that typically contain disulfide bonds. Trx-1 can also be found to be more oxidized when additional disulfide bonds are formed (43). ADAM17 has two CXXC motifs, but only in the disintegrin and

cysteine-rich domains (44, 45), and these motifs could be reduced by extracellular Trx-1.

Previous studies have shown that intracellular Trx-1 plays a crucial role in the ROS scavenging (43, 46) and that ROS and thiol isomerases are involved in the regulation of ADAM proteolytic activity (7-10, 47-50). Therefore, we investigated whether ADAM17 activity could be modulated by Trx-1 using a standard approach in a cell model of HB-EGF shedding coupled with an AP reporter assay (7, 25). We found that the transient overexpression of Trx-1-HA in HEK293 cells treated with PMA or EGF negatively modulated the activity of ADAM17 (Figure 6 and 7, respectively). The presence of ROS scavengers or the inhibition of cell surface oxidoreductases has previously been shown to prevent HB-EGF cleavage and LPS-induced ADAM17 activity (7, 10). Furthermore, we may also consider that ADAM17 cytoplasmic domain can solely function as an anchor domain to recruit Trx-1, which can regulate the redox state of cysteine residues in target proteins near or in the membrane, for instance in lipid rafts (50, 51).

In summary, the present study has demonstrated that Trx-1 is a novel interaction partner of the ADAM17 cytoplasmic domain and suggests that Trx-1 is a candidate that could be involved in the regulation of ADAM17 activity.

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*Acknowledgments* – We thank Dr. Axel Ullrich and Dr. Michael R. Freeman for kindly donation of ADAM17-HA and HB-EGF-HA vectors, respectively; and Dr. Luis E. Soares Neto, for Trx-1 antibody gift, and Dr. Carl Blobel for mEF cells donation.

## 4 DISCUSSÃO

### Trx-1: nova parceira de interação da ADAM17

É sabido que a porção citoplasmática das ADAMs varia muito em sua composição e quantidade de aminoácidos, sendo rica em resíduos de serina e prolina, com sequências consenso RXXPXXP e PXXPXR que possibilitam a interação desse domínio com outras proteínas. No artigo apresentado no Capítulo 1 (item 3.1), o papel do domínio citoplasmático da ADAM17 na modulação da sua atividade proteolítica foi investigado. Os resultados revelaram a Trx-1 como nova parceira de interação do domínio citoplasmático (*Figure 1*) e esse ligante foi capaz de modular a ativação da ADAM17 sob diferentes estímulos.

A superexpressão do domínio citoplasmático da ADAM17, contendo uma cauda FLAG na região N-terminal (*Table* 1), foi realizada em células HEK293 e comprovada pela técnica de *Imunnoblotting* (IB) (*Figure* 1A). Para identificação dos parceiros de interação foi utilizada cromatografia líquida acoplada à espectrometria de massas (LC-MS/MS) que revelou, dentre outras proteínas, a Trx-1 como nova parceira de interação do domínio citoplasmático da ADAM17 (*Figure* 1A). A interação entre Trx-1 e a ADAM17 completa (contendo os domínios extracelulares e transmembrana, além do citoplasmático) também foi avaliada por Co-imunoprecipitação (Co-IP) e comprovada por IB (*Figure* 1A, painel inferior). O peptídeo TAFQEALDAAGDK da Trx-1 teve seu espectro de massas validado manualmente (*Figure* 1B) e células HeLa, superexpressando a ADAM17 completa ou o domínio citoplasmático, foram marcadas pela técnica de Imunofluorescência para comprovar a co-localização entre ADAM17 recombinante e Trx-1 endógena, usando microscopia confocal de fluorescência (*Figure* 1C).

O domínio citoplasmático da ADAM17 pode ser fosforilado em seus resíduos de serina (S), treonina (T) e tirosina (Y). Foram descritos como sítios de fosforilação na porção citoplasmática da ADAM17 os resíduos de T735, S791 e S819 (DÍAZ-RODRIGUEZ *et al.*, 2002; FAN *et al.*, 2003; XU & DERYNCK, 2010). O tratamento com PMA ou outros ativadores, como fatores de crescimento, choque osmótico e luz UV promove a fosforilação desses resíduos, o que pode alterar a interação do domínio com seus ligantes. Considerando esses aspectos, avaliou-se a interação da Trx-1 quanto à dependência ou não da fosforilação dos resíduos do domínio citoplasmático da ADAM17. Os dados obtidos nesse estudo mostraram o aumento da fosforilação na S819 e a diminuição da fosforilação na S791 na presença do PMA (*Figure* 2 e *Table* 2), corroborando com os resultados do estudo de FAN *et al.* (2003). Comparando o peptídeo da Trx-1 na presença e ausência do PMA (*Table* 2) acredita-se que a ligação da Trx-1 ao domínio citoplasmático é independente do estado de fosforilação dos resíduos de S791 e S819.

Para explorar a interação entre o domínio citoplasmático da ADAM17 e a Trx-1, foram realizados experimentos complementares por ensaio de ligação em fase sólida (*Figure* 3), ressonância magnética nuclear (*Figure* 4) e ligação covalente entre resíduos de lisina (*chemical cross-linking)* analisados por LC-MS/MS. Os experimentos confirmaram a formação de um complexo entre as duas proteínas estudadas. O ensaio de ligação covalente usando o reagente DSS e analisado por MS revelou a formação de ligações entre lisinas de peptídeos intermoleculares, confirmados no espectro validado na figura 5A do artigo. A partir desses resultados, e em colaboração com o Dr. Paulo Oliveira e seu aluno Rodrigo Honorato, foram gerados modelos baseados na restrição de distância. A distância máxima entre as lisinas foi determinada pela região de interação permitida pelo reagente da ligação

química (DSS), mostrados nas figuras 5B e 5C do artigo. É importante ressaltar que nesse modelo o sítio ativo da Trx-1 ($Cys^{32}$-Gly-Pro-$Cys^{35}$) não se localiza na interface de interação com a ADAM17, ficando livre para interagir com outras proteínas e exercer sua atividade de oxiredutase (*Figure* 5C).

Para avaliar o papel da Trx-1 na modulação da atividade da ADAM17, células HEK293 expressando estavelmente HB-EGF-AP foram transfectadas com Trx-1-HA e ativadas com PMA ou EGF por 1h para avaliar os níveis de ativação da ADAM17 endógena. Trabalhos anteriores, utilizando o ensaio repórter da atividade de fosfatase alcalina como medida indireta da atividade de ADAM17, mostraram que o HB-EGF é um alvo específico dessa metaloprotease, sem contribuição expressiva do efeito da ADAM10 ou outras MMPs (SAHIN *et al.*, 2004; WILLEMS *et al.*, 2010).

É interessante notar que WILLEMS *et al.* (2010) mostraram a interação da ADAM17 com uma tiol-isomerase, a proteína PDI (*protein disulfide isomerase*), que apresenta domínios CXXC em sua molécula. A PDI modula negativamente a ADAM17 por se ligar ao domínio extracelular dessa metaloprotease, diferentemente do mecanismo sugerido em nosso estudo para a interação com a Trx-1, onde a ligação ocorre no domínio citoplasmático.

Além disso, outra classe de metaloprotease também tem sido relatada sendo modulada pela atividade da Trx-1. A MMP-2 teve sua atividade diminuída em células de neuroblastoma (FARINA *et al*., 2001), enquanto a MMP-9, em células de câncer de mama, teve um aumento de expressão seguido de desregulação do balanço entre MMP-9/TIMP-1 (seu inibidor natural), resultando no aumento da capacidade de invasão dessa linhagem na presença de Trx-1 superexpressa (FARINA *et al*., 2011).

Nossos resultados indicam que a superexpressão de Trx-1 modula a atividade de ADAM17 tanto sob a ativação pleiotrópica do PMA quanto sob a ação fisiológica do EGF (*Figure* 6 e 7).. Por sua vez, a Trx-1 além de atuar na própria atividade da ADAM17, poderia modular o estado oxidativo de proteínas-alvo na região próxima à membrana ou em microdomínios, como os *lipid rafts,* onde já foi descrita a presença tanto da ADAM17 quanto da Trx-1 (TELLIER *et al.*, 2006; HARA *et al.*, 2007). Esse resultado abre novas perspectivas sobre uma possível função do domínio citoplasmático, na ancoragem ou mesmo no recrutamento da Trx-1.

## 5. CONCLUSÃO

A análise dos parceiros de interação do domínio citoplasmático da ADAM17 demonstrou que a Trx-1 é um novo ligante desse domínio. Além disso, quando a ADAM17 endógena foi ativada pelo PMA ou EGF, ocorreu a modulação negativa da sua atividade na presença de Trx-1 superexpressa. Estudos buscando os parceiros da Trx-1, usando mutantes da Trx-1 e RNA de interferência (RNAi) já estão em andamento em nosso grupo, a fim de explorar e entender os possíveis mecanismos de atuação da Trx-1 na modulação da ADAM17.

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# 7. APÊNDICES

## 7.1 Artigos

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PLOS ONE

# Novel Processed Form of Syndecan-1 Shed from SCC-9 Cells Plays a Role in Cell Migration

**Annelize Z. B. Aragão[1☯], Marília Belloni[1☯], Fernando M. Simabuco[1], Mariana R. Zanetti[1], Sami Yokoo[1], Romênia R. Domingues[1], Rebeca Kawahara[1], Bianca A. Pauletti[1], Anderson Gonçalves[1], Michelle Agostini[2], Edgard Graner[2], Ricardo D. Coletta[2], Jay W. Fox[3], Adriana F. Paes Leme[1]***

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**Abstract**

The extracellular milieu is comprised in part by products of cellular secretion and cell surface shedding. The presence of such molecules of the sheddome and secretome in the context of the extracellular milieu may have important clinical implications. In cancer they have been hypothesized to play a role in tumor growth and metastasis. The objective of this study was to evaluate whether the sheddome/secretome from two cell lines could be correlated with their potential for tumor development. Two epithelial cell lines, HaCaT and SCC-9, were chosen based on their differing abilities to form tumors in animal models of tumorigenesis. These cell lines when stimulated with phorbol-ester (PMA) showed different characteristics as assessed by cell migration, adhesion and higher gelatinase activity. Proteomic analysis of the media from these treated cells identified interesting, functionally relevant differences in their sheddome/secretome. Among the shed proteins, soluble syndecan-1 was found only in media from stimulated tumorigenic cells (SCC-9) and its fragments were observed in higher amount in the stimulated tumorigenic cells than stimulated non-tumorigenic cells (HaCaT). The increase in soluble syndecan-1 was associated with a decrease in membrane-bound syndecan-1 of SCC-9 cells after PMA stimuli. To support a functional role for soluble syndecan-1 fragments we demonstrated that the synthetic syndecan-1 peptide was able to induce cell migration in both cell lines. Taken together, these results suggested that PMA stimulation alters the sheddome/secretome of the tumorigenic cell line SCC-9 and one such component, the syndecan-1 peptide identified in this study, was revealed to promote migration in these epithelial cell lines.

**Citation:** Aragão AZB, Belloni M, Simabuco FM, Zanetti MR, Yokoo S, et al. (2012) Novel Processed Form of Syndecan-1 Shed from SCC-9 Cells Plays a Role in Cell Migration. PLoS ONE 7(8): e43521. doi:10.1371/journal.pone.0043521

**Editor:** Effie C. Tsilibary, National Center for Scientific Research Demokritos, Greece

**Received** March 14, 2012; **Accepted** July 23, 2012; **Published** August 15, 2012



**Funding:** This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (470567/2009-0) and grants and fellowships from Fundação de Amparo à Pesquisa do Estado de São Paulo (2009/52833-0; 2009/18301-1; 2010/09642-7; 2010/19278-0). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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### Introduction

Oral cancer is one of the most common malignancies worldwide and despite improvements in diagnosis and treatment, the overall survival rate for advanced patients has not been significantly improved over the last three decades [1]. Indeed, the lack of biomarkers avoids prognostic prediction or specific treatment for oral squamous cell carcinomas (OSCC), the most common presentation of oral cancer.

New approaches on clinical proteomics, such as secretome-based analysis, have been developed to identify novel biomarkers. Secretome/sheddome is a proteomic area that allows the analysis of a dynamic extracellular environment including secreted, released, degraded or shed proteins [2–4]. Soluble proteins in the extracellular milieu can have specific functions and can induce a variety of responses that are still not predictable, for instance, notch, E-cadherin and CD44 are known candidates for potential outside-in signal transduction [5–8]. These fragments can carry over conserved sequences that can regulate autocrine and paracrine targets [9].

In order to evaluate the differences between the secretome/sheddome of normal and tumorigenic cells, two epithelial cell lines, HaCaT and SCC-9, were treated with phorbol-ester (PMA). Here we showed that PMA stimulation induced distinct migration, adhesion and gelatinase activity as well as differences in the secretome/sheddome. Components in the media such as soluble and fragments of syndecan-1 were found mainly in stimulated tumorigenic cells. Syndecans are known family of cell surface proteoglycans that play regulatory roles in many biological processes, including migration, proliferation, wound healing, inflammation, angiogenesis and tumorigenesis [10,11]. The role of syndecans in tumor progression may vary with tumor stage and type [10]. In squamous cell carcinoma, the reduction of syndecan-1 expression is correlated with the progression of carcinogenesis [12], histological grade of malignancy [13], tumor size and the mode of invasion [14]. Furthermore, we also demonstrated evidence that the fragment of syndecan-1 identified was able to induce cell migration.

Simabuco et al. Molecular Cancer 2014, 13:24
http://www.molecular-cancer.com/content/13/1/24

MOLECULAR CANCER

RESEARCH Open Access

# ADAM17 mediates OSCC development in an orthotopic murine model

Fernando Moreira Simabuco[1,2], Rebeca Kawahara[1], Sami Yokoo[1], Daniela C Granato[1], Lucas Miguel[1], Michelle Agostini[4], Annelize ZB Aragão[1], Romênia R Domingues[1], Isadora L Flores[1,3], Carolina CS Macedo[1,3], Ricardo Della Coletta[3], Edgard Graner[3] and Adriana Franco Paes Leme[1*]

## Abstract

**Background:** ADAM17 is one of the main sheddases of the cells and it is responsible for the cleavage and the release of ectodomains of important signaling molecules, such as EGFR ligands. Despite the known crosstalk between ADAM17 and EGFR, which has been considered a promising targeted therapy in oral squamous cell carcinoma (OSCC), the role of ADAM17 in OSCC development is not clear.

**Method:** In this study the effect of overexpressing ADAM17 in cell migration, viability, adhesion and proliferation was comprehensively appraised *in vitro*. In addition, the tumor size, tumor proliferative activity, tumor collagenase activity and MS-based proteomics of tumor tissues have been evaluated by injecting tumorigenic squamous carcinoma cells (SCC-9) overexpressing ADAM17 in immunodeficient mice.

**Results:** The proteomic analysis has effectively identified a total of 2,194 proteins in control and tumor tissues. Among these, 110 proteins have been down-regulated and 90 have been up-regulated in tumor tissues. Biological network analysis has uncovered that overexpression of ADAM17 regulates Erk pathway in OSCC and further indicates proteins regulated by the overexpression of ADAM17 in the respective pathway. These results are also supported by the evidences of higher viability, migration, adhesion and proliferation in SCC-9 or A431 cells *in vitro* along with the increase of tumor size and proliferative activity and higher tissue collagenase activity as an outcome of ADAM17 overexpression.

**Conclusion:** These findings contribute to understand the role of ADAM17 in oral cancer development and as a potential therapeutic target in oral cancer. In addition, our study also provides the basis for the development of novel and refined OSCC-targeting approaches.

## Introduction

ADAM17 (*A Disintegrin And Metalloproteinase*) or TACE (TNF-alpha Converting Enzyme) is a surface membrane associated protein responsible for the cleavage of several membrane proteins, which is a biological cell process called shedding [1,2]. Among the shed proteins, ADAM17 releases ectodomains of important signaling molecules such as TNF-α, TGF-α, EGF, HB-EGF and VEGFR2 and adhesion molecules, such as L-selectin, syndecans, CAMs (cell adhesion molecules) and cadherins [1,3]. ADAM17 expression, likewise other ADAM family members, is up-regulated in many types of cancers, correlating with tumor progression and aggressiveness [4]. The molecules that are shed by ADAM17 are mostly signaling molecules that regulate cell proliferation, survival, migration and invasion properties associated with malignant cells resulted mainly from the crosstalk between ADAM17 and epidermal growth factor receptor (EGFR) [1,5]. Interestingly, EGFR is a widely studied oncogene in head and neck tumors [6] and agents targeting EGFR have emerged as a potential adjuvant therapy for OSCC [7,8]. Then, despite the close relationship between ADAM17 and EGFR, it is still not clear the role that ADAM17 plays in oral cancer development. Metalloproteinases are particular important in oral cancer progression, and squamous cell carcinoma of head and neck has been classified as the fifth most common type of cancer in the world [9].

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PLOS ONE

# Integrated Proteomics Identified Up-Regulated Focal Adhesion-Mediated Proteins in Human Squamous Cell Carcinoma in an Orthotopic Murine Model

**Daniela C. Granato[1], Mariana R. Zanetti[1], Rebeca Kawahara[1], Sami Yokoo[1], Romênia R. Domingues[1], Annelize Z. Aragão[1], Michelle Agostini[2,3], Marcelo F. Carazzolle[1], Ramon O. Vidal[1], Isadora L. Flores[1,2], Johanna Korvala[5], Nilva K. Cervigne[2], Alan R. S. Silva[2], Ricardo D. Coletta[2], Edgard Graner[2], Nicholas E. Sherman[4], Adriana F. Paes Leme[1]***

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## Abstract

Understanding the molecular mechanisms of oral carcinogenesis will yield important advances in diagnostics, prognostics, effective treatment, and outcome of oral cancer. Hence, in this study we have investigated the proteomic and peptidomic profiles by combining an orthotopic murine model of oral squamous cell carcinoma (OSCC), mass spectrometry-based proteomics and biological network analysis. Our results indicated the up-regulation of proteins involved in actin cytoskeleton organization and cell-cell junction assembly events and their expression was validated in human OSCC tissues. In addition, the functional relevance of talin-1 in OSCC adhesion, migration and invasion was demonstrated. Taken together, this study identified specific processes deregulated in oral cancer and provided novel refined OSCC-targeting molecules.

**Citation:** Granato DC, Zanetti MR, Kawahara R, Yokoo S, Domingues RR, et al. (2014) Integrated Proteomics Identified Up-Regulated Focal Adhesion-Mediated Proteins in Human Squamous Cell Carcinoma in an Orthotopic Murine Model. PLoS ONE 9(5): e98208. doi:10.1371/journal.pone.0098208

**Editor:** Christina Lynn Addison, Ottawa Hospital Research Institute, Canada

**Received** October 15, 2013; **Accepted** April 30, 2014; **Published** May 23, 2014



**Funding:** This work was supported by FAPESP Grants: 2009/54067-3, 2010/19278-0, 2011/22421-2, 2009/53839-2 and 2011/02267-9; CNPq Grants: 470567/2009-0, 470549/2011-4, 301702/2011-0, 470268/2013-1, and 505413/2013-2; and Science without Borders Program Grant MEC/MCTI/CAPES/CNPq/FAPs 109/2012. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Oral cancer is one of the most common malignancies worldwide [1,2] and the third most frequent cancer, with a 5-year survival rate less than 50% [3]. The development of oral squamous cell carcinoma (OSCC) requires the accumulation of several genetic alterations that are affected by genetic predisposition and environmental conditions such as tobacco, alcohol, chronic inflammation and viral infection [4]. Because cancer is a complex and multifactorial disease, exploring the molecular pathways involved in this process is necessary to achieve successful treatment of each specific case and improve the understanding of pathogenesis [4–6].

Therefore, in order to investigate the mechanisms for oral cancer development, this study focused on analyzing the differential expression of proteins and peptides in OSCC compared to normal tissue using an orthotopic murine model, which recapitulates the local tumor microenvironment [7,8]. We used a two-step approach by first injecting SCC-9 cells and the respective control cells in the tongues of immunodeficient mice to induce tumor development. After 20 days, tumor and control tissues were isolated, and extracted proteins and peptides were analyzed using mass spectrometry, followed by validation using human OSCC tissues. We demonstrated that the strategies used here enabled the identification of up-regulated focal adhesion-mediated proteins for OSCC, such as filamins A and B, catenin alpha-1 and talin-1 as potential proteins involved in OSCC development.

## Materials and Methods

### Cell culture

The human OSCC cell line SCC-9 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured as recommended. SCC-9 cells are originated from human squamous carcinoma from the tongue. The HaCaT cells, an immortalized but not transformed epithelial cell line [9], was maintained in DMEM containing 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 5% $CO_2$ air atmosphere. HaCaT cells are human keratinocytes originated from skin. Control cells were used to assure that all the animals were subjected to the same procedures. Human Epidermoid Carcinoma A431 (epidermoid carcinoma cell line originated from skin) was grown in Roswell Park Memorial Institute (RPMI) −1640 medium supplemented with 10% FBS and antibiotics at 37°C in a 5% $CO_2$ air atmosphere. Metastatic SCC-9 cells were isolated from lymph nodes (LN) originating the cell line SCC-9 LN1 [10]. This cell line was cultured as recommended for SCC-9 cells.

Journal of Cellular Physiology

# INTERACTOME REVEALS MOLECULAR NETWORKS OF HUMAN THIOREDOXIN-1



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| Journal: | *Journal of Cellular Physiology* |
| Manuscript ID: | Draft |
| Wiley - Manuscript type: | Original Research Article |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | Aragão, Annelize; Laboratório Nacional de Biociências, LNBio, CNPEM, Laboratório de Espectrometria de Massas<br>Granato, Daniela; Laboratório Nacional de Biociências, LNBio, CNPEM, Laboratório de Espectrometria de Massas<br>Yokoo, Sami; Laboratório Nacional de Biociências, LNBio, CNPEM, Laboratório de Espectrometria de Massas<br>Kawahara, Rebeca; Laboratório Nacional de Biociências, LNBio, CNPEM, Laboratório de Espectrometria de Massas<br>Ruivo, Mateus; Laboratório Nacional de Biociências, LNBio, CNPEM, Laboratório de Espectrometria de Massas<br>Paes Leme, Adriana; Laboratório Nacional de Biociências, |
| Key Words: | Thioredoxin-1, Protein-protein interaction, Proteomics |
| | |

# 8. ANEXOS

**DECLARAÇÃO**

Declaro para os devidos fins que o conteúdo de minha tese de Doutorado intitulada A Tioredoxina-1 é uma nova parceira de interação do domínio citoplasmático da ADAM17 e participa da sua modulação

( ) não se enquadra no § 4º do Artigo 1º da Informação CCPG 002/13, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

( x ) CIBio – Comissão Interna de Biossegurança , projeto No. AFPL 14, Instituição: Laboratório Nacional de Biociências – LNBio-CNPEM (ABTLuS).

( ) CEUA – Comissão de Ética no Uso de Animais , projeto No. _____, Instituição: _____.

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Aluno: Annelize Zambon Barbosa Aragão

Orientador: Adriana Franco Paes Leme

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Carimbo e assinatura

Prof. Dr. MARCELO LANCELLOTTI
Presidente da Comissão Interna de Biossegurança
Instituto de Biologia - UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

Carimbo e assinatura

Uso exclusivo da CIBio:

Número de projeto / processo: AFPL 1.4

**Formulário de encaminhamento de projetos de pesquisa para análise pela CIBio - Comissão Interna de Biossegurança da ABTLuS – Associação Brasileira de Tecnologia de Luz Síncrotron**

Título do projeto: Estudo da regulação da ADAM17 recombinante pela determinação dos sítios de fosforilação do domínio citoplasmático e de seus ligantes em células normais e de câncer

Pesquisador responsável: Adriana Franco Paes Leme

Experimentador: Annelize Zambon Barbosa Aragão

Nível do treinamento do experimentador: [ ]-Iniciação científica, [ ]-mestrado, [ x ]-doutorado, [ ]-doutorado direto, [ ]-pós-doutorado, [ ]-nível técnico, [ ]-outro, especifique:_____

Resumo do projeto:

As ADAMs (metaloproteinases de mamíferos compostas dos domínios metaloproteinase, tipo-disintegrina e rico em cisteínas, tipo fator de crescimento epidermal, domínio transmembrana e citoplasmático) apresentam-se com expressão aumentada em tumores malignos, como o carcinoma de células escamosas de cabeça e pescoço, que é considerado o quinto mais comum no mundo. Considerando as vias que essas proteases podem modular, essas proteases estão muito associadas à tumorigênese e progressão de tumor. Têm sido apontadas como reguladoras chaves dos mecanismos de sinalização celular, pois são responsáveis pela liberação de ectodomínios de proteínas de superfície celular mediando a transdução de sinal entre GPCR e EGFR. Está claro que existe essa via tripla de ativação e que as ADAMs funcionam como efetores da sinalização mediada pela GPCR. Entretanto, pouco se sabe sobre os mecanismos de ativação das ADAMs via domínio citoplasmático e as vias que essas proteases podem regular. Dessa forma, esse projeto se propõe a estudar os mecanismos de ativação das ADAMs via fosforilação do domínio citoplasmático e o seu papel na regulação das vias de sinalização em queratinócitos normais e cancerosos (carcinoma oral de células escamosas). Com essa finalidade, será realizada transfecção e super-expressão de ADAM17 recombinante em células normais e cancerosas. Para determinação dos sítios de fosforilação no domínio citoplasmático, a ADAM17 recombinante será recuperada por imunoprecipitação com anticorpo anti-FLAG e as proteínas que se ligarem às ADAMs serão eluídas com peptídeo FLAG. A determinação dos sítios de fosforilação e a identificação dos ligantes do domínio citoplasmático serão realizadas por espectrometria de massas e a validação dos alvos da ADAM17 será realizada por *western blot*. Além disso, ensaios de viabilidade, migração e invasão para comparação do efeito da super-expressão da ADAM recombinante no comportamento das células também serão realizados. O conhecimento dos ligantes e das vias de sinalização e regulação das ADAMs podem elucidar alvos atrativos para o desenho de drogas que modulem a ação dos seus ligantes.

A CIBio analisou este projeto em reunião realizada no dia: 16.12.10 .

Parecer final: [X]-projeto aprovado, [ ]-projeto recusado, [ ]-projeto com deficiências, favor comentários abaixo:

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Jörg Kobarg

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