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Catherine Anne Parr-Sturgess, David James Rushton, Edward Thomas Parkin. Ectodomain shedding of the Notch ligand Jagged1 is mediated by ADAM17 but is not a lipid raft-associated event. *Biochemical Journal*, 2010, 432 (2), pp.283-294. 10.1042/BJ20100321 . hal-00535581

HAL Id: hal-00535581

<https://hal.science/hal-00535581>

Submitted on 12 Nov 2010

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Ectodomain shedding of the Notch ligand Jagged1 is mediated by ADAM17 but is not a lipid raft-associated event

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Running Title: Raft-independent ADAM17-mediated shedding of Jagged1

Abbreviations; ADAM, a disintegrin and metalloproteinase; APP, amyloid precursor protein; CPM, carboxypeptidase M; CTF, C-terminal fragment; Dll, Delta-like ligand; DSL, Delta/Serrate/Lag-2; GPI, glycosylphosphatidylinositol; HEK, human embryonic kidney; hTfR, human transferrin receptor; ICD, intracellular domain; MBS, Mes-buffered saline; NICD, Notch intracellular domain; PBS, phosphate-buffered saline; RIP, regulated intramembrane proteolysis; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

SYNOPSIS

Notch signaling is an evolutionarily conserved pathway involved in cell fate specification. The initiating event in this pathway is the binding of a Notch receptor to a Delta/Serrate/Lag-2 (DSL) ligand on neighbouring cells triggering the proteolytic cleavage of Notch within its extracellular juxtamembrane region; a process known as proteolytic 'shedding' and catalyzed by members of the ADAM (a disintegrin and metalloproteinase) family of enzymes. Jagged1 is a Notch binding DSL ligand which is also shed by an ADAM-like activity raising the possibility of bi-directional cell-cell Notch signaling. In the current study we have unequivocally identified the sheddase responsible for shedding Jagged1 as ADAM17, the activity of which has previously been shown to be localized within specialized microdomains of the cell membrane known as 'lipid rafts'. However, we have shown that replacing the transmembrane and cytosolic regions of Jagged1 with a glycosylphosphatidylinositol (GPI) anchor, thereby targeting the protein to lipid rafts, did not enhance its shedding. Furthermore, the Jagged1 holoprotein, its ADAM-cleaved C-terminal fragment, and ADAM17 were not enriched in raft preparations devoid of contaminating non-raft proteins. We have also demonstrated that wild-type Jagged1 and a truncated polypeptide-anchored variant lacking the cytosolic domain were subject to similar constitutive and phorbol ester-regulated shedding. Collectively these data demonstrate that Jagged1 is shed by ADAM17 in a lipid raft-independent manner and that the cytosolic domain of the former protein is not a pre-requisite for either constitutive or regulated shedding.

Key words: ADAM17, amyloid precursor protein, glycosylphosphatidylinositol, Jagged1, Notch, rafts

INTRODUCTION

Proteolytic cleavage of proteins within their juxtamembrane region and subsequent ectodomain release, a process known as 'shedding', represents a common molecular mechanism for regulating the biological activity of a range of cell surface proteins [1]. The key enzymes that regulate ectodomain shedding are members of the a disintegrin and metalloproteinase (ADAM) family of zinc metalloproteinases, type I integral membrane proteins characterized by a conserved domain structure consisting of an N-terminal signal sequence followed by a prodomain, a metalloproteinase domain, a disintegrin domain with a cysteine-rich region, an epidermal growth factor-like domain, a transmembrane domain, and a cytoplasmic domain [1]. ADAM-mediated ectodomain shedding constitutes the rate-limiting step in an emerging group of molecular signaling pathways involving the regulated intramembrane proteolysis (RIP) of cell surface membrane proteins [1].

The original RIP paradigm is that of Notch signaling, an evolutionarily conserved pathway with a key role in various developmental processes, adult homeostasis, and stem cell maintenance [2]. Regulated intramembrane proteolysis of Notch is initiated by interactions of membrane-associated Notch receptors and Delta/Serrate/Lag-2 (DSL) ligands on neighbouring cells [2]. This receptor-ligand interaction results in a conformational change in the Notch receptor facilitating ADAM-mediated shedding of its extracellular domain (S2-cleavage). Both ADAM10 and ADAM17 have been implicated in Notch receptor shedding in a cell and context-dependant manner [3-5]. The resultant Notch membrane-associated fragment is subject to further proteolytic cleavage by a presenilin-dependent γ -secretase complex [6] resulting in the translocation of the soluble Notch intracellular domain (NICD) into the nucleus where it interacts with the transcriptional factor CSL (CBF1/RBPJk in mammals, Su(H) in flies, and LAG1 in worms) to activate downstream target genes.

To date, five DSL ligands have been identified in mammals; Dll(Delta-like ligand)1, Dll3, Dll4, Jagged1, and Jagged2 [2]. Certain DSL ligands, like Notch receptors, are themselves subject to RIP. The Dll1 homologue, Delta, was originally shown to be cleaved by Kuzbanian (an ADAM10 homologue) in *Drosophila* [7] and Dll1 itself appears to be processed by ADAM10 in mammalian systems [8], although ADAMs 9, 17, and 12 have now also been implicated in this process [9]. Furthermore, Dll1 and Jagged2 generate intracellular domains (ICDs) via a presenilin-dependant mechanism [10]. The ICDs generated from Dll1 and another DSL ligand, Jagged1, participate in cell signaling and the regulation of gene transcription [11].

The ectodomain shedding activities of a select few ADAMs are thought to be concentrated within specialized cholesterol- and sphingolipid-enriched microdomains at the cell surface known as 'lipid rafts' which participate in a range of processes including signaling, protein trafficking, neurotransmission, and endocytosis [12]. Specifically, ADAM19-mediated shedding of Neuregulin-1 occurs in neuronal lipid rafts [13] and the ADAM17-mediated shedding of a range of substrates is purported to be concentrated in rafts with the mature form of the enzyme itself being concentrated within these structures [14]. Furthermore, it has been inferred, from the fact that lipid raft disruption using cholesterol-lowering drugs alters the shedding of a range of proteins, that these shedding events must occur within such membrane microdomains [15-19].

In terms of a physiological rationale for Jagged1 shedding it is possible that the event may simply be a pre-requisite for Jagged1 intracellular domain generation and subsequent nuclear signaling [11]. However, it is becoming increasingly apparent that the soluble Jagged1

ectodomain also possesses important physiological functions. A truncated soluble form of Jagged1 participates in endothelial cell differentiation repressing the function of its transmembrane counterpart [20, 21] and in the regulation of keratinocyte differentiation [22]. Repression of transmembrane Jagged1 signaling by the soluble form of the protein has also been implicated in the induction of hematopoietic stem cell self-renewal [23] and the clonal expansion of neural crest stem cells [24]. More recently the soluble forms of both Dll1 and Jagged1 have been shown to promote *in vivo* tumorigenicity of fibroblasts [25].

In the current study we sought to unequivocally identify the sheddases responsible for Jagged1 shedding and to determine whether the activity of this enzyme was associated with lipid rafts. We have shown that ADAM17, but not ADAM10, is responsible for the shedding of over-expressed Jagged1 in human embryonic kidney (HEK) cells. Levels of endogenous Jagged1 in most cell types are too low to accurately monitor the small proportion of the protein shed from the cell surface [11]. However, Jagged1 levels are significantly enhanced in a range of cancers [26]. Consequently, we used the human cervical carcinoma cell line, CaSki, to confirm the identity of ADAM17 as the key enzyme responsible for the shedding of endogenous Jagged1. Furthermore, by artificially targeting Jagged1 to lipid rafts, we have demonstrated that the enzyme activity responsible for shedding the protein is not concentrated in these microdomains. In agreement with this observation we have also shown that the Jagged1 holoprotein, ADAM-cleaved Jagged1 C-terminal fragment, and ADAM17 itself are not enriched in lipid raft fractions from HEK cells. Finally, by using a truncated Jagged1 construct which retained the transmembrane polypeptide region but which lacked the cytosolic domain of the wild-type protein, we have confirmed that the latter domain does not directly regulate either constitutive or phorbol ester-regulated Jagged1 shedding.

EXPERIMENTAL

Materials

FLAG-tagged Jagged1 constructs were synthesized by Epoch Biolabs (Missouri City, Texas, U.S.A.). Coding DNA sequences, preceded by a 5' Kozak sequence were cloned into BamH1/EcoRV digested mammalian expression vector pIRESHyg (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). Anti-Jagged1 C-terminal polyclonal antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, U.S.A.). Anti-Jagged1 ectodomain and anti-human transferrin receptor polyclonal antibodies were from R&D Systems Europe Ltd. (Abingdon, U.K.). Anti-FLAG M2 and anti-actin monoclonal antibodies were from Sigma-Aldrich Company Ltd. (Gillingham, U.K.). Anti-APP 6E10 monoclonal antibody was from Cambridge Bioscience Ltd. (Cambridge, U.K.). Anti-ADAM10 C-terminal and anti-ADAM17 C-terminal polyclonal antibodies were from Merck Chemicals (Nottingham, U.K.). Anti-caveolin polyclonal antibody was from BD Biosciences (Oxford, U.K.). All other materials, unless otherwise stated, were from sources previously noted [27].

Cell culture

All cell culture reagents were purchased from Lonza Ltd. (Basel, Switzerland). HEK cells were cultured in Dulbecco's modified Eagle's medium supplemented with 25 mM glucose, 4 mM L-glutamine, 10% (v/v) foetal bovine serum, penicillin (50 U/ml), streptomycin (50 µg/ml), and fungizone (2.5 µg/ml). The human cervical carcinoma cell line, CaSki, was cultured in Roswell Park Memorial Institute-1640 medium with the same supplements. All cells were maintained at 37°C in 5% CO₂ in air.

Stable DNA transfections

HEK cells were stably transfected with FLAG-tagged Jagged1 constructs in pIRESHyg or the empty vector control. Plasmids (8 µg) were linearized using AhdI before being subjected to ethanol precipitation, complexed with Lipofectamine 2000 (Invitrogen, Paisley, U.K.), and transfected into cells. Recombinant cells were selected using 150 µg/ml of Hygromycin B (Invitrogen, Paisley, U.K.).

Transient DNA and small interfering RNA (siRNA) transfections

HEK cells (80% confluence) were transiently transfected with 8 µg of ADAM10 or ADAM17 expression plasmids (or the corresponding empty vector control) using Lipofectamine 2000 (Invitrogen, Paisley, U.K.) according to the manufacturer's instructions. Cells were used 36 h post-transfection. For siRNA treatments HEK and CaSki cells were grown to 40% and 80% confluence, respectively, in antibiotic-free growth medium and transfected with 100 nM siRNA duplexes (Eurogentec Ltd., Southampton, U.K.) using oligofectamine (Invitrogen, Paisley, U.K.) or Dharmafect (ABgene Ltd., Epsom, U.K.) for HEK and CaSki cells, respectively, according to the manufacturers' instructions. Three siRNA sequences were tested for each protein. ADAM10 siRNA sense sequences were 5'-GCCAAGUUCUUGAGAAGAAdTdT-3', 5'-GAUAUCCAGUCAUGUAAAAdTdT-3', and 5'-CUGGAAUUAUACUGUUCAdTdT-3'. ADAM17 siRNA sense sequences were 5'-GCUUGUUCAUCGAGUGAAAdTdT-3', 5'-CUACGAUAUUCUCUCUUUAdTdT-3', and 5'-GGAUGUAAUUGAACGAUUUdTdT-3'. Control cells were subjected to mock transfection with scramble siRNA. Cells were used 48 h post-transfection.

Treatment of cells and protein extraction

Cells were grown to confluence in 75 cm³ culture flasks and rinsed three times with reduced serum medium (10 ml) (OptiMEM; Invitrogen, Paisley, U.K.). A fresh 10 ml of OptiMEM was then conditioned on cells for the indicated periods. Ilomastat and phorbol myristate acetate (PMA) were added to cells at the indicated concentrations alongside control flasks treated with an equal volume of dimethyl sulphoxide vehicle. Media for immunoblot analyses were harvested, centrifuged at 10,000 g for 10 min to remove cell debris, and concentrated 50-fold using Vivaspin 6 centrifugal concentrators (Sartorius, Epsom, U.K.). For analysis of cell-associated proteins, cells were washed with phosphate-buffered saline (PBS; 20 mM Na₂HPO₄, 2 mM NaH₂PO₄, 0.15 M NaCl, pH 7.4) and scraped from the flasks into fresh PBS (10 ml). Following centrifugation at 500 g for 5 min, cell pellets were lysed in 100 mM Tris, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% (v/v) Nonidet P40, 10 mM 1,10-phenanthroline, pH 7.4.

Protein assay, alkaline phosphatase assay, and enzyme pre-treatments

Protein was quantified using bicinchoninic acid [28] in a microtitre plate with bovine serum albumin (BSA) as a standard. Alkaline phosphatase activity was assayed as described previously [27]. Conditioned media samples were deglycosylated using an enzymatic deglycosylation kit (Europa Bioproducts Ltd., Ely, U.K.) according to the manufacturer's instructions.

Isolation of lipid rafts

HEK cells grown to confluence in a 75 cm³ culture flask were washed *in situ* with 2 X 10 ml of Mes-buffered saline (MBS; 25 mM Mes, 0.15 M NaCl, pH 6.5) before being scraped into 10 ml of the same buffer and pelleted by centrifugation at 1500 g for 5 min. All subsequent manipulations were performed at 4°C using pre-cooled solutions. The cell pellet was resuspended in 2 ml of 0.5% (v/v) Triton X-100 in MBS and homogenisation was achieved by passing the resuspension ten times through a 21G needle. The sample was then diluted with

an equal volume of 80% (w/v) sucrose in MBS and aliquots (1 ml) were layered under a discontinuous sucrose gradient consisting of 2 ml each of 5% and 35% (w/v) sucrose in MBS. The sucrose gradients were then subjected to ultracentrifugation at 100,000 *g* for 18 h at 4°C in an SW50.1 rotor (Beckman Instruments). Individual fractions were harvested from the bottom to the top of the gradient. For direct comparison of raft and non-raft fractions on a protein-protein basis, raft fractions from the sucrose gradients were diluted 5-fold with PBS and centrifuged at 100,000 *g* for 90 min. The supernatant was discarded and the raft pellet was resuspended in PBS to the same protein concentration as the non-raft fraction.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoelectrophoretic blot analysis

Samples were mixed with a half volume of reducing electrophoresis sample buffer and boiled for 3 min. Proteins were resolved by SDS-PAGE using either 5-15% or 7-17% polyacrylamide gradient gels and transferred to Immobilon P polyvinylidene difluoride (PVDF) membranes as previously described [29]. Anti-Jagged1 C-terminal and anti-Jagged1 ectodomain antibodies were used at dilutions of 1:2000 and 1:500, respectively. Anti-ADAM10 C-terminal, anti-ADAM17 C-terminal, and anti-FLAG M2 antibodies were used at a dilution of 1:1000. Anti-caveolin and anti-actin antibodies were used at a dilution of 1:5000. Anti-APP 6E10 antibody was used at a dilution of 1:2500. Reconstituted anti-human transferrin receptor antibody was used at a final concentration of 0.1 µg/ml. Bound antibody was detected using peroxidase-conjugated secondary antibodies (Sigma-Aldrich Company Ltd., Gillingham, U.K. and R&D Systems Europe Ltd., Abingdon, U.K.) in conjunction with enhanced chemiluminescence detection reagents (Perbio Science Ltd, Cramlington, U.K.).

Statistical analysis

All data are presented as the means ± SD. Data were subjected to statistical analysis via Student's *t*-test. Levels of significance are indicated in the figure legends.

RESULTS

Stable expression of Jagged1 constructs in HEK cells

Levels of endogenous Jagged1 are low in most non-cancerous cell lines making detection of the minor shed pool of the protein impossible. Therefore, in order to study the molecular mechanisms governing Jagged1 shedding, we designed and stably over-expressed three Jagged1 DNA constructs in HEK cells; WT-JAG1, GPI-JAG1, and ΔICD-JAG1 (Fig. 1A). All three constructs contained a FLAG epitope-tag positioned C-terminal to Gly-56 to facilitate the initial differentiation of the over-expressed proteins from any potential endogenously expressed Jagged1. WT-JAG1 differed from full-length human Jagged1 only by the insertion of the said intramolecular FLAG epitope-tag. GPI-JAG1 consisted of the first 1073 residues of WT-JAG1 (including the FLAG-tag) fused to the twenty-four residue GPI anchor signal sequence of human carboxypeptidase M (CPM). ΔICD-JAG1 consisted of the first 1101 residues of WT-JAG1 (including the FLAG-tag) but was truncated immediately C-terminal to the transmembrane domain, thereby lacking the cytosolic domain of its wild-type counterpart. Immunodetection of Jagged1 in HEK cell lysates using the anti-JAG1 ectodomain antibody (Fig. 1B) revealed a single band at approximately 185 kDa in WT-JAG1-transfected cells consistent with previous observations relating to the expression of the glycosylated human holoprotein in mammalian cells [11, 30]. The same antibody detected slightly smaller bands in lysates from GPI-JAG1- and ΔICD-JAG1-transfected cells consistent with the truncated nature of these constructs. The identity of these bands as Jagged1 was confirmed using anti-FLAG-M2 antibody. In contrast, immunodetection of

Jagged1 in cell lysates using the anti-JAG1 C-terminal antibody only detected the holoprotein in WT-JAG1-transfected cell lysates and not in mock cell lysates (demonstrating a lack of significant endogenous Jagged1 expression in HEK cells) or in GPI-JAG1 and Δ ICD-JAG1 transfected cell lysates (consistent with the fact that the latter two constructs lacked the cytosolic domain of the protein). The anti-JAG1 C-terminal antibody also detected a band at approximately 20 kDa in WT-JAG1-transfected cell lysates consistent with previous observations relating to the ADAM-cleaved C-terminal fragment (CTF) of human Jagged1 [11]. As expected, the CTF was not generated in cells expressing GPI-JAG1 or Δ ICD-JAG1.

WT-JAG1 ectodomain shedding is responsive to phorbol ester and ilomastat treatment in a manner identical to that of the amyloid precursor protein

In order to gain some preliminary insight into the possible identity of the Jagged1 sheddase(s) in HEK cells, we compared the shedding of the protein to that of endogenously expressed amyloid precursor protein (APP) which has previously been shown to be shed predominantly by ADAM10 and/or ADAM17 [31, 32]. Specifically, we compared the stimulation of APP and Jagged1 shedding by the phorbol ester, phorbol myristate acetate (PMA) and the inhibition of shedding by the ADAM inhibitor, ilomastat (Fig. 2). The extents of WT-JAG1 and APP shedding stimulation by PMA were statistically indistinguishable (3.14 ± 0.74 -fold and 3.55 ± 0.47 -fold, respectively). Similarly, the extents of constitutive WT-JAG1 and APP shedding inhibition by ilomastat were also statistically indistinguishable ($89.51 \pm 18.16\%$ and $80.70 \pm 6.44\%$, respectively). It should be noted that PMA caused only a minor, statistically insignificant, enhancement of total protein levels in cell lysates (1.19 ± 0.12 -fold) and no change in total protein levels in conditioned media. As such, general changes in protein synthesis caused by PMA could not have been responsible for the large increases observed in the shedding of both APP and Jagged1. Consequently, these data indicated that WT-JAG1 and APP may have been shed by the same, or closely related, members of the ADAM family of zinc metalloproteinases.

Over-expressed Jagged1 is shed by ADAM17 but not ADAM10 in HEK cells

As over-expressed WT-JAG1 was shed from HEK cells in a manner indistinguishable from that of endogenous APP, we hypothesized that ADAM10 and/or ADAM17 may be the predominant Jagged1 sheddase(s) in this cell line. In order to test this hypothesis, we transiently transfected HEK-WT-JAG1 cells with cDNA encoding these ADAMs and examined the resultant effects on Jagged1 shedding (Figs. 3A-3C). Immunodetection of ADAM10 in lysates prepared from mock- and ADAM10-transfected cells (Fig. 3A) confirmed successful protein over-expression with the enzyme being detected as two prominent bands at 98 kDa and 70 kDa corresponding to the immature (prodomain containing) and mature (prodomain lacking) forms, respectively [33]. Transient over-expression of ADAM10 did not, however, enhance WT-JAG1 shedding (Figs. 3A and 3C). Transient transfections were also performed using cDNAs encoding ADAM9 and ADAM15 (which are thought to regulate the shedding of various proteins indirectly by shedding ADAM10 from the cell [34, 35]) but did not reveal any changes in Jagged1 shedding (data not shown). When ADAM17 was transiently over-expressed (Fig. 3B) the enzyme was detected as a doublet at approximately 110 kDa consistent with previous reports [36-38]. In contrast to ADAMs 9, 10 and 15, the transient over-expression of ADAM17 enhanced WT-JAG1 shedding 2.14 ± 0.61 -fold (Figs. 3B and 3C). Note that the intensity of the JAG1 ectodomain band in media from mock-transfected cells differs between Figs. 3A and 3B as the experiments were performed independently and developed on separate immunoblots. In a further experiment, HEK-WT-JAG1 cells were transfected with siRNAs targeted against endogenous human ADAM10 or ADAM17 and the shedding of Jagged1 into conditioned

media was subsequently monitored (Figs. 3D and 3E) (three different siRNA duplexes were tested for each protein). The siRNA duplexes targeted against ADAM10 depleted endogenous levels of the protein by $60.14 \pm 10.16\%$ and did not affect ADAM17 protein levels (representative immunoblot shown in Fig. 3D). The depletion of endogenous ADAM10 did not, however, affect WT-JAG1 shedding (Fig. 3E). In contrast, the siRNA duplexes targeted against ADAM17 decreased levels of the endogenous protein by $73.21 \pm 5.62\%$ without affecting levels of ADAM10 (representative immunoblot shown in Fig. 3D) and, concomitantly, resulted in an $81.72 \pm 13.90\%$ decrease in WT-JAG1 shedding (Fig. 3E).

ADAM17 is responsible for shedding endogenous Jagged1 in CaSki cells

As there remained the possibility that the over-expressed Jagged1 in HEK cells might be subject to proteolysis by ADAMs not normally involved in the shedding of the endogenous protein, we next sought to confirm that depleting endogenous ADAM17 levels also depleted shedding of endogenous Jagged1. Unfortunately, levels of endogenous Jagged1 in most non-cancerous cell lines are low or undetectable making detection of the minor shed pool of the protein impossible [11]. Furthermore, in order to perform siRNA depletion of endogenous ADAMs it was essential that any cell line used expressed suitable levels of endogenous ADAM10 and ADAM17 thereby further limiting the choice of cell model. However, the expression of various elements of the Notch signaling pathway is increased in a number of cancers and cancerous cell lines [26]. In particular, high levels of endogenous Jagged1 have previously been detected in the human cervical carcinoma cell line, CaSki [39]. Having ascertained that this cell line also expressed high levels of ADAM10 and ADAM17, CaSki cells were transfected with siRNAs targeted against endogenous human ADAM10 or ADAM17 (Fig. 4). The siRNA duplexes targeted against ADAM10 depleted endogenous levels of the protein by $95.88 \pm 2.73\%$ and did not affect ADAM17 protein levels (representative immunoblot shown in Fig. 4A). As a positive control for reduced ADAM10 shedding activity we demonstrated that the ADAM10 siRNA treatment resulted in an $80.77 \pm 4.65\%$ reduction in the level of APP shed from CaSki cells (Fig. 4B) demonstrating that the enzyme is the key constitutive APP sheddase in this cell line. However, the depletion of ADAM10 levels, had no effect on the shedding of endogenous Jagged1 (Fig. 4C). The siRNA duplexes targeted against ADAM17 decreased levels of the endogenous protein $97.23 \pm 4.16\%$ without affecting ADAM10 levels (representative immunoblot shown in Fig. 4A) and, concomitantly, resulted in a $94.38 \pm 12.12\%$ decrease in WT-JAG1 shedding (Fig. 4C). Depletion of ADAM17 levels in CaSki cells did not affect the shedding of APP (Fig. 4B). Collectively, these results demonstrate that ADAM17 is the key enzyme responsible for shedding endogenous Jagged1.

GPI-JAG1 but not WT-JAG1 is targeted to lipid rafts in HEK cells

Having identified ADAM17 as a key Jagged1 sheddase, we next sought to determine whether the shedding activity was enriched in lipid rafts by artificially targeting the Jagged1 substrate to these structures in HEK cells. This was achieved by replacing the transmembrane and cytosolic domains of the wild-type protein with a GPI anchor (see GPI-JAG1 construct in Fig. 1A). HEK cells stably expressing WT-JAG1 or GPI-JAG1 were solubilised in Triton X-100 at 4°C and rafts isolated by buoyant sucrose density-gradient centrifugation as described in the Experimental section. In both HEK-WT-JAG1 and HEK-GPI-JAG1 cells most of the total cellular protein was effectively solubilised and subsequently located in fractions 1-3 of the sucrose gradients (Fig. 5A). The position of rafts in fractions 5 and 6 of the sucrose gradients was determined by assaying GPI-anchored alkaline phosphatase activity (Fig. 5B) and by immunoblotting for caveolin (Fig. 5C). As a non-raft protein marker we also immunoblotted the sucrose gradient fractions for human transferrin receptor (hTfR) [40] which was detected

as a 90 kDa band exclusively present in the non-raft regions of the sucrose gradients (Fig. 5D). Immunoblotting for Jagged1 using the anti-JAG1 ectodomain antibody revealed that the wild-type protein was completely excluded from rafts whereas 65% of GPI-JAG1 was targeted to these structures (Fig. 5E) in a near identical manner to that of endogenous GPI-anchored alkaline phosphatase (Fig. 5B). Collectively, these data clearly demonstrate that GPI-JAG1 but not WT-JAG1 was effectively targeted to lipid rafts in HEK cells.

Lipid raft targeting and the cytosolic domain of Jagged1 are not pre-requisites for constitutive or regulated shedding of the protein

It has previously been proposed that lipid rafts are focal points for the ADAM17-mediated shedding of various proteins [14]. It has also been hypothesized that phorbol ester-regulated shedding may be explained by protein kinase C-mediated modification of the cytosolic domains of sheddase substrate proteins with associated conformational changes leading to exposure of the cleavage site [41]. Consequently, we sought to determine whether the constitutive or phorbol ester-regulated shedding of raft-targeted GPI-JAG1 or that of our Δ ICD-JAG1 construct (lacking the cytosolic domain of the wild-type protein) differed significantly from the shedding of WT-JAG1.

In order to determine the extent of constitutive shedding of the various constructs, we resolved equal volumes (20 μ l) of lysate and media (conditioned for 5 h) samples alongside each other on the same gels. The results (Fig. 6A) clearly show that the relative levels of Jagged1 in lysates and media for all three constructs were the same. Multiple immunoblots of the type shown in Fig. 6A were quantified and the data adjusted to compensate for differences in the total volumes of lysates and conditioned media. The resultant data were combined with the results from ilomastat inhibition experiments in order to determine the proportion of each Jagged1 construct shed from the cell surface over a 5 h period in the absence and presence of inhibitor (Fig. 6B). The results demonstrated that $6.57 \pm 1.23\%$ of WT-JAG1 was shed from cells after 5 h. The constitutive shedding of GPI-JAG1 ($7.78 \pm 1.05\%$) and Δ ICD-JAG1 ($6.98 \pm 1.35\%$) did not differ significantly from that of WT-JAG1 and the constitutive shedding of all three constructs was effectively inhibited by ilomastat.

In a further experiment we compared the phorbol ester-regulated shedding of GPI-JAG1 and Δ ICD-JAG1 to that of WT-JAG1 (Figs. 6C and 6D). Following PMA treatment no significant difference was detected between the regulated shedding of WT-JAG1 and either GPI-JAG1 or Δ ICD-JAG1 (Fig. 6D). Interestingly, the PMA-regulated shedding of both GPI-JAG1 and Δ ICD-JAG1 was more effectively inhibited by ilomastat than that of the wild-type protein. As was the case for WT-JAG1 expressing cells (Fig. 2) no significant changes in the total protein levels in lysates or conditioned media from GPI-JAG1 or Δ ICD-JAG1 cells were detected following PMA treatment.

Of note, the shed forms of WT-JAG1, GPI-JAG1, and Δ ICD-JAG1 were of an identical molecular weight and all three shed proteins were similarly glycosylated as determined by enzymatic deglycosylation (see Supplementary Data Fig. S1). The removal of the transmembrane and/or the cytosolic domains of Jagged1 did not, therefore, appear to grossly impact the post-translational modification of the protein.

Collectively, these data demonstrate that neither raft localisation nor the cytosolic domain of Jagged1 are pre-requisites for the constitutive or regulated shedding of the protein.

ADAM17 and the Jagged1 CTF are not enriched in lipid rafts isolated from HEK cells

Whilst the WT-JAG1 holoprotein was not present in lipid rafts isolated from HEK cells (Fig. 5E) and targeting GPI-JAG1 to rafts did not affect shedding of the protein (Fig. 6), there remained the possibility that WT-JAG1 in rafts may be shed very efficiently by ADAM17 such that steady-state levels of the holoprotein in these structures would be undetectable. In

order to address this possibility we examined raft preparations for the presence of the ADAM-cleaved Jagged1 CTF and also for the presence of ADAM17 itself. Lipid rafts were prepared from both mock- and ADAM17 transiently-transfected HEK-WT-JAG1 cells as described in the Experimental section. Having confirmed that the raft containing fractions from the gradients were not contaminated with non-raft proteins (by immunoblotting for hTfR) the samples were immunoblotted for ADAM17. The results (Fig. 7A) show that no endogenous ADAM17 was present in the raft fractions (5 and 6) from HEK cells with only a very minor fraction of the enzyme being located in rafts from ADAM17 transiently-transfected cells. When rafts were concentrated and compared on a protein-protein basis with the soluble non-raft fraction of cells, ADAM17 was found to be enriched in the latter fraction (Fig. 7B). The Jagged1 CTF was found to be exclusively present in non-raft fractions (Figs. 7C and 7D).

DISCUSSION

In this work we show conclusively that ADAM17 is the key sheddase responsible for the generation of the Jagged1 soluble ectodomain but that raft localisation and the Jagged1 cytosolic domain are not prerequisites for the constitutive or phorbol ester-regulated shedding of the protein.

When WT-JAG1 was over-expressed in HEK cells the holoprotein was detected at approximately 185 kDa (Fig. 1B) consistent with previous reports relating to the expression of the glycosylated human holoprotein in mammalian cells [11, 30]. The ADAM-cleaved Jagged1 CTF was detected only in cells over-expressing WT-JAG1 and, as expected, was not generated from the GPI-JAG1 and Δ ICD-JAG1 proteins. Interestingly, levels of the CTF in cell lysates were reduced by only 10% following incubation with ilomastat for 5 h (data not shown) compared to a 90% reduction of soluble Jagged1 levels in conditioned media (Fig. 6B). These data indicate that the Jagged1 CTF accumulates in cells throughout the entire culture period as the ectodomain of the protein is shed continuously i.e. the CTF has a long half-life. Consequently, the subsequent 5 h incubation in the presence of ilomastat, despite nearly completely blocking Jagged1 shedding, made only a limited impact on the total cell-associated levels of the CTF. Whilst only a small proportion ($6.57 \pm 1.23\%$) of total WT-JAG1 was shed during a 5 h incubation period (Fig. 6B) the longer term effect of shedding would be to generate high steady-state levels of cell-associated Jagged1 CTF. A smaller peptide fragment was also observed just beneath the Jagged1 CTF following the over-exposure of immunoblots consistent with the generation of a Jagged1 ICD as previously described [11]. It is interesting to note that, given the apparent long half-life of the Jagged1 CTF, some factor other than ectodomain shedding must be rate-limiting in the subsequent γ -secretase-mediated generation of the Jagged1 ICD.

The fact that Jagged1 shedding from HEK cells was responsive to phorbol ester and ilomastat treatment in a manner identical to that of APP (Fig. 2) led us to investigate whether the key APP sheddases, ADAM10 and ADAM17 were also responsible for Jagged1 shedding. Our results (Fig. 3) from both ADAM over expression and siRNA experiments, for the first time, directly implicate ADAM17 (but not ADAM10) as the predominant constitutive Jagged1 sheddase in HEK cells over-expressing Jagged1. Levels of endogenous Jagged1 in most cell types are too low to accurately monitor the small proportion of the protein shed from the cell surface [11]. However, Jagged1 levels are significantly enhanced in a range of cancers [26]. In particular, high levels of endogenous Jagged1 have previously been detected in the human cervical carcinoma cell line, CaSki [39]. We found that this cell line also expressed high levels of endogenous ADAM10 and ADAM17 permitting the use of ADAM siRNA to deplete these endogenous enzymes. This particular cancer cell line was also remarkably

amenable to siRNA transfection resulting in an extremely efficient depletion of endogenous ADAMs allowing us to demonstrate that ADAM17 is also a key enzyme in the shedding of endogenous Jagged1 (Fig. 4). Collectively, these data vindicate previous suggestions as to the identity of the Jagged1 sheddase made by LaVoie & Selkoe [11] on the basis that the shedding of the over-expressed protein from Chinese Hamster Ovary cells was inhibited by TAPI-1 (which demonstrates a degree of ADAM17 specificity) but not by TIMP1 (which demonstrates some specificity towards ADAM10).

The ectodomain shedding activities of several ADAMs are thought to be concentrated within lipid rafts [12]. Specifically, ADAM19-mediated shedding of Neuregulin-1 occurs in neuronal lipid rafts [13] and the ADAM17-mediated shedding of a range of substrates is purported to be concentrated in rafts with the mature form of the enzyme itself being concentrated within these structures [14]. Given the possible involvement of rafts in the ADAM17-mediated shedding of cell surface proteins we hypothesized that these microdomains might be focal points for Jagged1 shedding. This hypothesis was tested in two ways; first by artificially targeting Jagged1 to rafts and second by examining rafts for the presence of ADAM17 and the ADAM-cleaved Jagged1 CTF. In the first approach we designed a Jagged1 construct (GPI-JAG1) in which all the amino acid residues of the wild-type protein C-terminal to Arg-1065 were replaced with the twenty-four residue GPI anchor signal sequence of human carboxypeptidase M. GPI-JAG1 was effectively targeted to rafts when expressed in HEK cells (Fig. 5) and its distribution in sucrose gradients mirrored that of a known GPI-anchored protein, alkaline phosphatase [42]. In contrast, the WT-JAG1 holoprotein was present exclusively in the non-raft region of gradients (Fig. 5). Despite the predominant raft localization of GPI-JAG1 its constitutive and regulated shedding were identical to those of its wild-type counterpart (Fig. 6) suggesting that the shedding activity of the Jagged1 sheddase (ADAM17) was not enriched in lipid rafts. Furthermore, our results also demonstrate that ADAM17 (mature and immature) was present, but not enriched in rafts, and that the ADAM-cleaved Jagged1 CTF was completely excluded from these structures (Fig. 7) indicating further that ADAM17-mediated shedding of Jagged1 is not a raft-dependent event. Two previous studies have shown that ADAM17 is not enriched in rafts [19, 43] with a third suggesting that the mature form of the enzyme is enriched in these structures [14]. It is notable that, in the latter study, raft preparations were not monitored for the presence of contaminating non-raft marker proteins such as hTfR [40] which was shown to be completely excluded from raft preparations in the current study (Fig. 5). Furthermore, the contaminant-free nature of rafts prepared from a range of cell lines and tissues using the current methodology has previously been extensively characterised by the corresponding author [33, 44-46]. Also of note is the fact that the samples employed by Tellier *et al.* [14] were snap frozen in liquid nitrogen prior to raft preparation; a process which we have found dramatically affects the raft/non-raft distribution of proteins.

It has been hypothesized previously that phorbol ester-regulated shedding may be explained by protein kinase C-mediated modification of the cytosolic domains of sheddase substrate proteins with associated conformational changes leading to exposure of the cleavage site [41]. In this respect we sought to determine whether the cytosolic domain of Jagged1 participated in the constitutive or regulated shedding of Jagged1 from the surface of HEK cells. We designed a construct, Δ ICD-JAG1, which retained the transmembrane polypeptide domain of the wild-type protein but which was truncated immediately C-terminal to this region, thereby lacking the wild-type cytosolic domain (Fig. 1A). When expressed in HEK cells, Δ ICD-JAG1 was constitutively shed in a manner identical to WT-JAG1 clearly showing that the cytosolic domain of the protein was not a pre-requisite for ADAM17-mediated constitutive shedding of

the protein (Fig. 6). Similarly, the extent of Jagged1 shedding stimulation by PMA was identical for both WT-JAG1 and Δ ICD-JAG1 (Fig. 6) indicating that modification of the Jagged1 cytosolic domain is not a pre-requisite for phorbol ester-regulated shedding. However, whilst the extent of both Δ ICD-JAG1 and GPI-JAG1 shedding stimulation by PMA did not differ from WT-JAG1, it is interesting to note that the regulated shedding of the former two constructs was more effectively inhibited than the latter by ilomastat (Fig. 6D). This observation raises the possibility that the removal of the Jagged1 cytosolic domain invokes regulated cleavage of the protein by enzymes distinct from those which shed the wild-type protein.

In the current study we have demonstrated unequivocally for the first time that Jagged1 is constitutively shed by ADAM17 but that the process does not occur in a raft-dependant manner. Similarly we have also shown that the cytosolic domain of Jagged1 is not a pre-requisite for the constitutive or regulated shedding of the protein. The involvement of Jagged1 in cell signaling may be considered biphasic in that both the soluble ectodomain [20-25] and the cytosolic domain of the protein have been implicated [11, 30]. In the current study we have developed two constructs (GPI-JAG1 and Δ ICD-JAG1) that yield identical levels of cell-associated holoprotein and that are shed identically to WT-JAG. However, only the latter construct generates a CTF making it possible, in the future, to delineate between cellular changes brought about by production of the soluble ectodomain and generation of the Jagged1 CTF. In this respect, we are currently in the process of expressing these constructs in a range of cancer-derived cell lines with a view to characterising the relative contributions of the Jagged1 ectodomain and CTF to the proliferation and invasiveness of these cells.

FUNDING

This work was supported by the North West Cancer Research Fund (grant code CR774).

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FIGURE LEGENDS

Figure 1. Stable expression of Jagged1 constructs in HEK cells

(A) WT-JAG1 is a type I transmembrane protein with C-terminal transmembrane (diagonal lines) and cytosolic (dots) domains, and an N-terminal signal sequence (black). A FLAG-tag was inserted downstream of Gly56. Numbers in parentheses are wild-type (untagged) Jagged1 numbering. GPI-JAG1 consists of the first 1073 residues of WT-JAG1 (including the FLAG-tag) fused to the twenty-four residue GPI anchor signal sequence of human carboxypeptidase M (grey and expanded sequence). The site of GPI anchor attachment is also indicated (*). Δ ICD-JAG1 consists of the first 1101 residues of WT-JAG1 (including the FLAG-tag) but is truncated immediately C-terminal to the transmembrane domain and, therefore, lacks the cytosolic domain of its wild-type counterpart. (B) HEK cells were stably transfected with plasmids encoding the various Jagged1 constructs (lane 1, mock; lane 2, WT-JAG1; lane 3, GPI-JAG1; lane 4, Δ ICD-JAG1), lysates were then prepared and immunoblotted with the antibodies indicated. Full-length (FL) and the C-terminal fragment (CTF) of Jagged1 are indicated by the arrowheads.

Figure 2. WT-JAG1 ectodomain shedding is responsive to phorbol ester and ilomastat treatment in a manner identical to that of the amyloid precursor protein

HEK cells stably transfected with WT-JAG1 were incubated for 5 h in OptiMEM (10 ml) in the absence or presence of PMA (1 μ M) or ilomastat (25 μ M). Conditioned media and cell lysates were subsequently processed and immunoblotted as described in the Experimental section. (A) Conditioned media were immunoblotted with anti-JAG1 ectodomain or anti-APP antibody 6E10. (B) Multiple anti-JAG1 ectodomain and anti-APP 6E10 immunoblots of conditioned media were quantified by densitometric analysis. Levels of shed proteins are expressed relative to the untreated controls. Results are means \pm S.D. (n=3). n.s. = not significant.

Figure 3. Over-expressed WT-JAG1 is shed by ADAM17 and not ADAM10 in HEK cells.

HEK cells stably transfected with WT-JAG1 were incubated for 5 h in OptiMEM (10 ml). Conditioned media and cell lysates were subsequently processed and immunoblotted as

described in the Experimental section. (A-C) Prior to conditioning the media, cells were transiently transfected with either expression vector alone (Mock), ADAM10, or ADAM17 DNA. Lysates and conditioned media from ADAM10 (A) and ADAM17 (B) transfected cells were immunoblotted with the antibodies indicated. (C) Multiple anti-JAG1 ectodomain immunoblots of conditioned media from DNA-transfected cells were quantified by densitometric analysis and results are shown as means \pm S.D. (n=3). (D and E) Prior to conditioning media, cells were transfected with control siRNA duplexes (Mock) or siRNAs derived from the coding sequences of ADAM10 or ADAM17. Lysates (D) and conditioned media (E) from mock, ADAM10 siRNA, and ADAM17 siRNA transfected cells were immunoblotted with the antibodies indicated. (E) Multiple anti-JAG1 ectodomain immunoblots of conditioned media from siRNA-transfected cells were quantified by densitometric analysis and results are shown as means \pm S.D. (n=3). n.s. = not significant; * = significant at $p \geq 0.05$; *** = significant at $p \geq 0.005$.

Figure 4. ADAM17 is responsible for shedding endogenous Jagged1 in CaSki cells.

Human cervical carcinoma, CaSki, cells were incubated for 10 h in OptiMEM (10 ml). Conditioned media and cell lysates were subsequently processed and immunoblotted as described in the Experimental section. (A-C) Prior to conditioning media, cells were transfected with control siRNA duplexes (Mock) or siRNAs derived from the coding sequences of ADAM10 or ADAM17. Lysates and conditioned media from mock, ADAM10 siRNA, and ADAM17 siRNA transfected cells were immunoblotted with the antibodies indicated. (B and C) Multiple anti-APP antibody 6E10 (B) and anti-JAG1 ectodomain immunoblots (C) of conditioned media from siRNA-transfected cells were quantified by densitometric analysis and results are shown as means \pm S.D. (n=3). n.s. = not significant; **** = significant at $p \geq 0.001$.

Figure 5. GPI-JAG1 but not WT-JAG1 is targeted to lipid rafts in HEK cells.

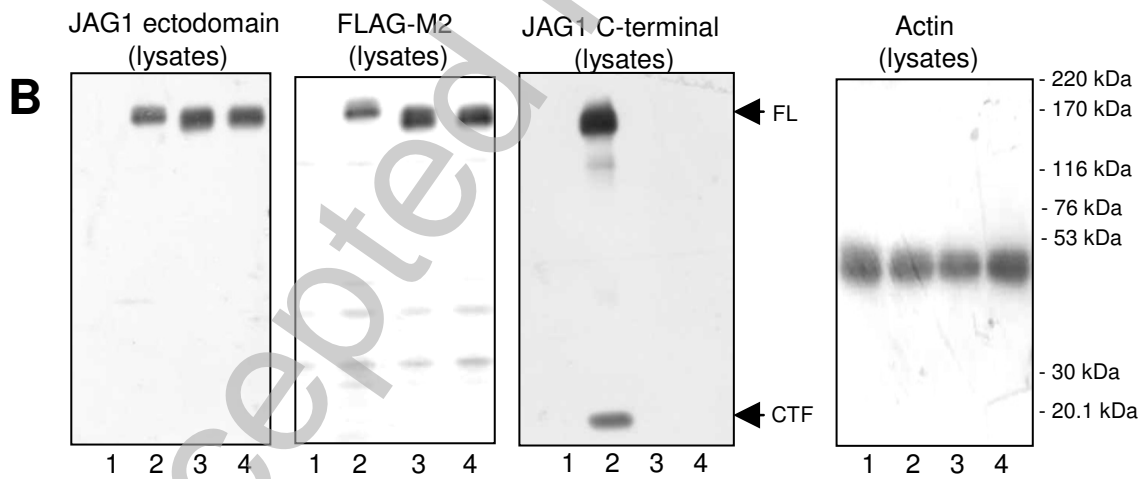
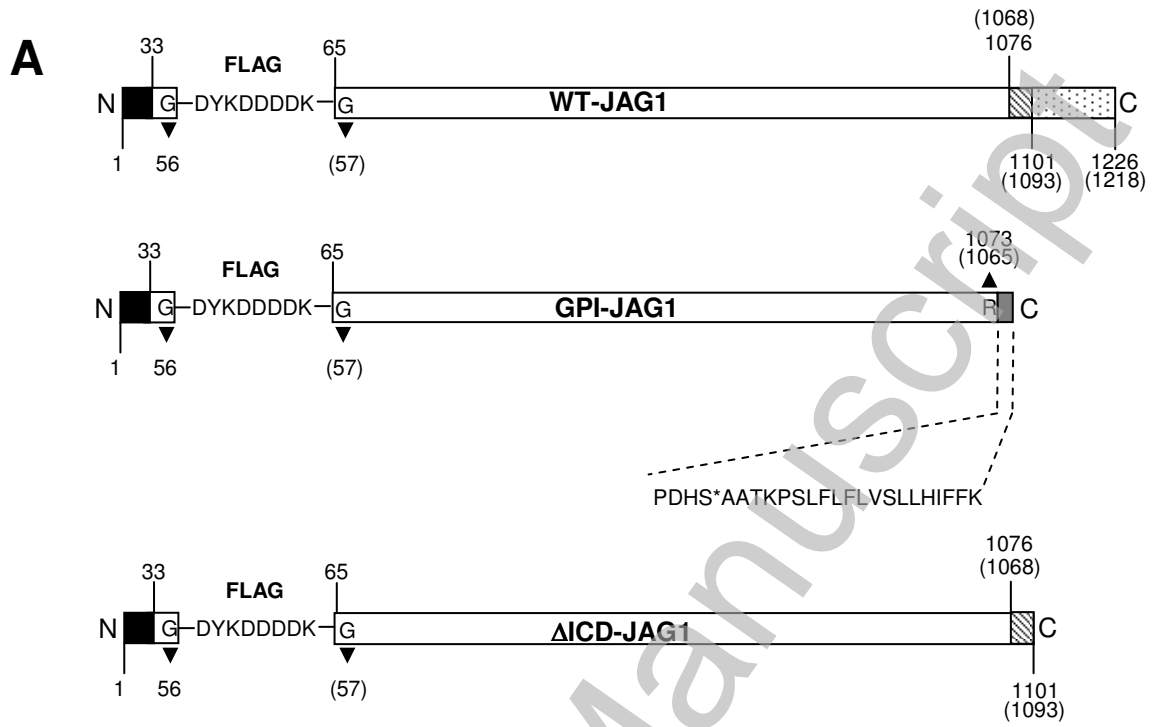
Lipid rafts were prepared by solubilisation of cells in Triton X-100 at 4°C with subsequent buoyant sucrose density-gradient centrifugation as described in the Experimental section. Individual fractions were harvested from the gradients (1, base of gradient; 9, top of gradient) and assayed for total protein (A) and alkaline phosphatase activity (B), or immunoblotted with (C) anti-caveolin, (D) anti-hTfR, and (E) anti-JAG1 ectodomain. Results are means \pm S.D. (n=3).

Figure 6. Constitutive and phorbol ester regulated shedding of Jagged1 constructs.

HEK cells stably transfected with WT-JAG1, GPI-JAG1, or Δ ICD-JAG1 were incubated for 5 h in OptiMEM (10 ml). Conditioned media and cell lysates were subsequently processed and immunoblotted as described in the Experimental section. (A) Protein from equal volumes (20 μ l) of lysates (L) and conditioned media (M) from HEK cells transfected with the indicated constructs were resolved on the same gels and immunoblotted with anti-JAG1 ectodomain antibody. (B) Percent of total Jagged1 protein (i.e. cell-associated + soluble) shed into conditioned media over a 5 h period in the absence or presence of ilomastat (25 μ M) adjusted to take into account total volumes of lysates and media. Results are means \pm S.D. (n=3). (C) Cells transfected with the indicated constructs were incubated for the 5 h time course in the absence or presence of PMA (1 μ M) or PMA (1 μ M) + ilomastat (25 μ M). Conditioned media were then immunoblotted with the anti-JAG1 ectodomain antibody. (D) Multiple anti-JAG1 ectodomain antibody immunoblots of the type shown in panel C were quantified by densitometric analysis. Levels of shed proteins are expressed relative to the untreated controls. Results are means \pm S.D. (n=3). n.s. = not significant; * = significant at $p \geq 0.05$; *** = significant at $p \geq 0.005$.

Figure 7. ADAM17 and the Jagged1 C-terminal fragment are not enriched in lipid rafts. HEK cells stably transfected with WT-JAG1 were used to prepare lipid rafts by solubilisation of cells in Triton X-100 at 4°C with subsequent buoyant sucrose density-gradient centrifugation as described in the Experimental section. Individual fractions were harvested from the gradients (1, base of gradient; 9, top of gradient). **(A)** Individual sucrose density-gradient fractions from HEK WT-JAG1 cells (top panel) or HEK WT-JAG1 cells transiently transfected with ADAM17 (bottom panel) were immunoblotted in order to detect ADAM17. **(B)** Equal amounts of protein from non-raft (NR) and raft (R) fractions of ADAM17 transiently transfected cells were immunoblotted in order to detect ADAM17. **(C)** Individual sucrose density-gradient fractions from HEK WT-JAG1 cells were immunoblotted in order to detect the Jagged1 C-terminal fragment. **(D)** Equal amounts of protein from non-raft (NR) and raft (R) fractions from HEK WT-JAG1 cells were immunoblotted in order to detect the Jagged1 holoprotein, Jagged1 C-terminal fragment, and caveolin.

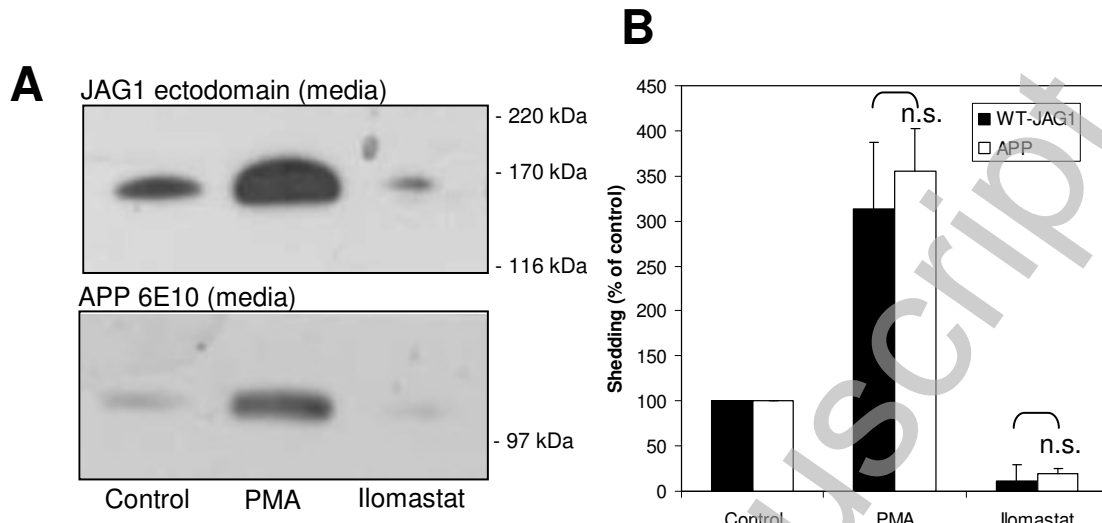
Figure 1.



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Figure 2.



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Figure 3.

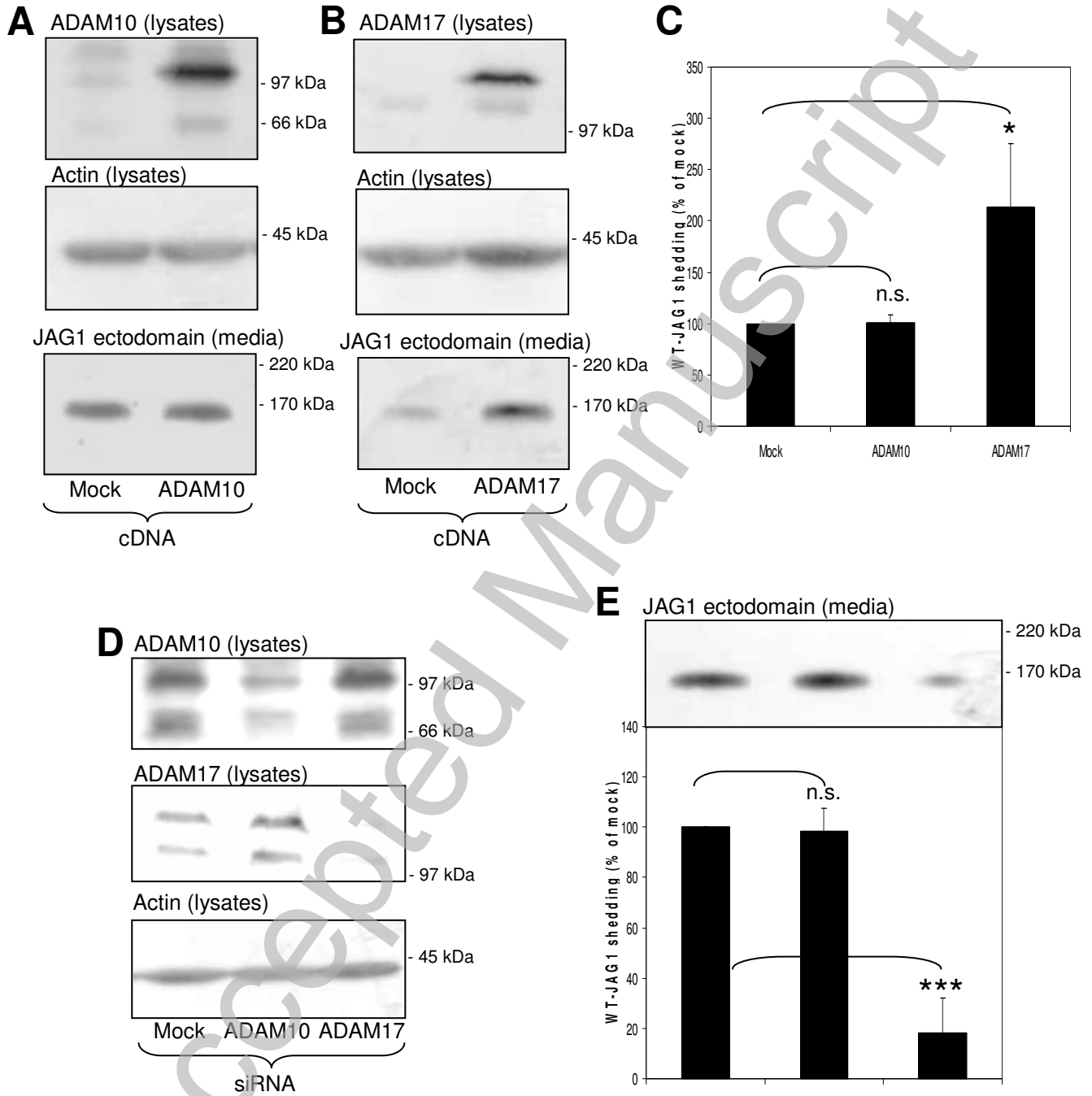
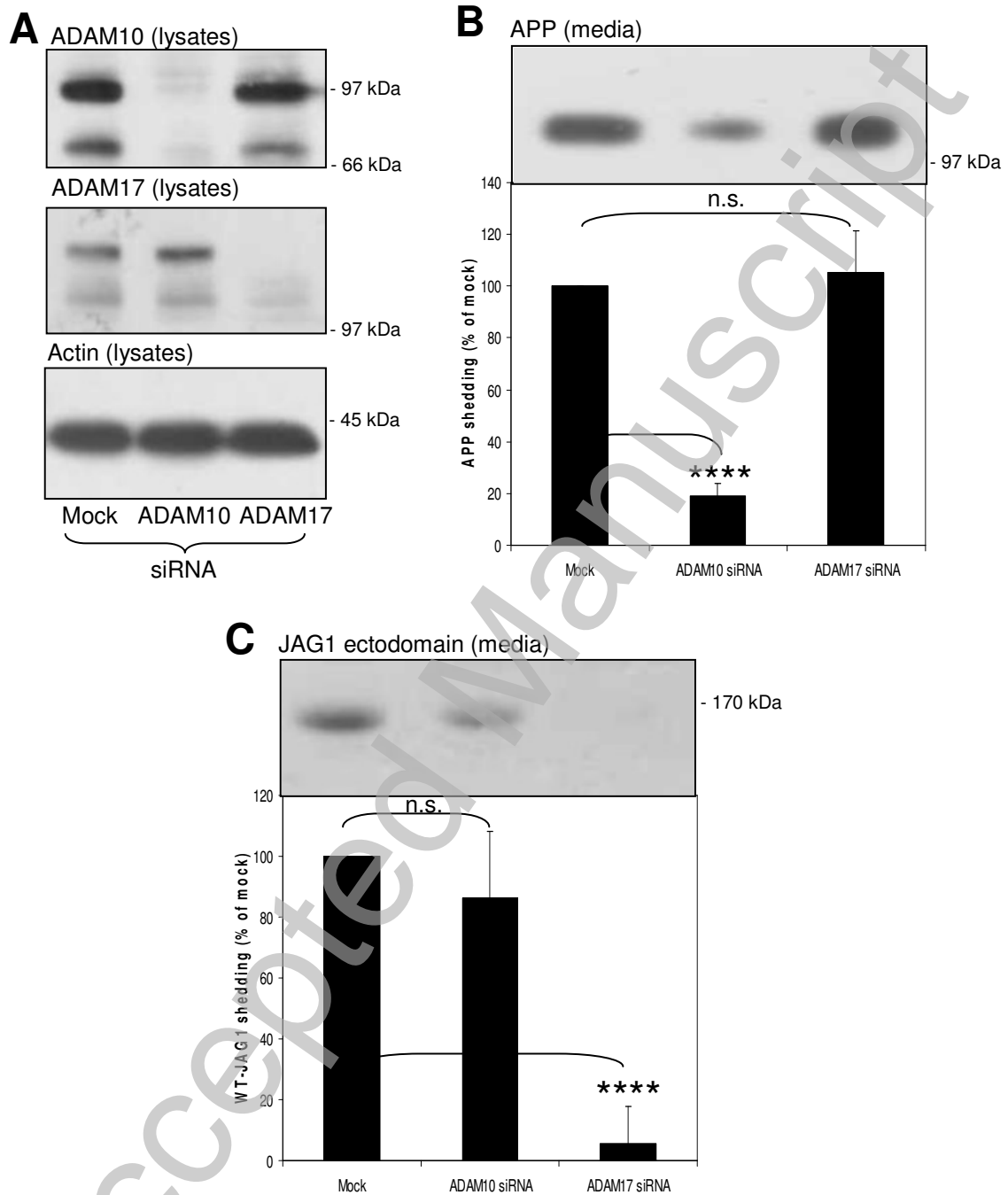
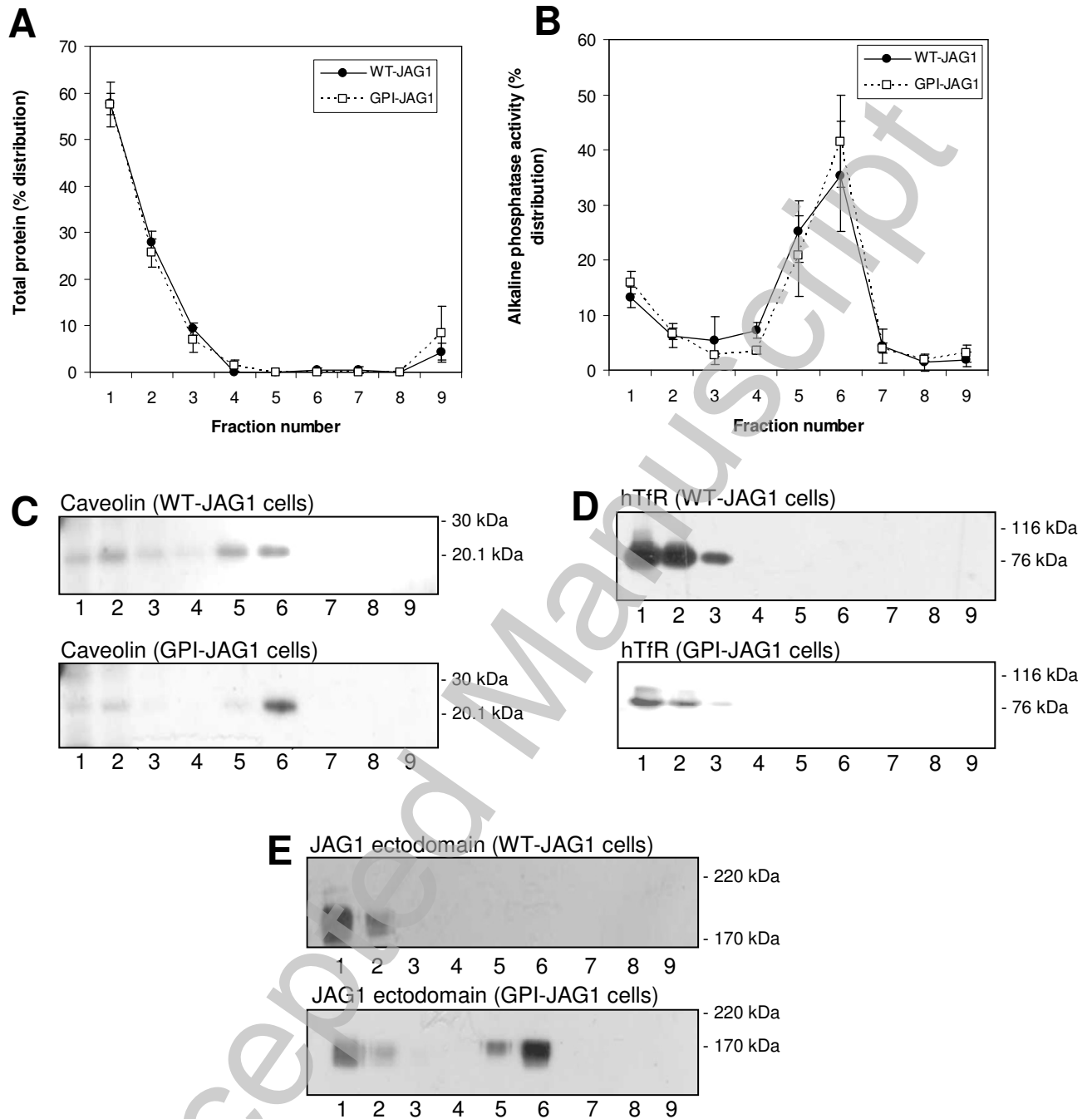


Figure 4.



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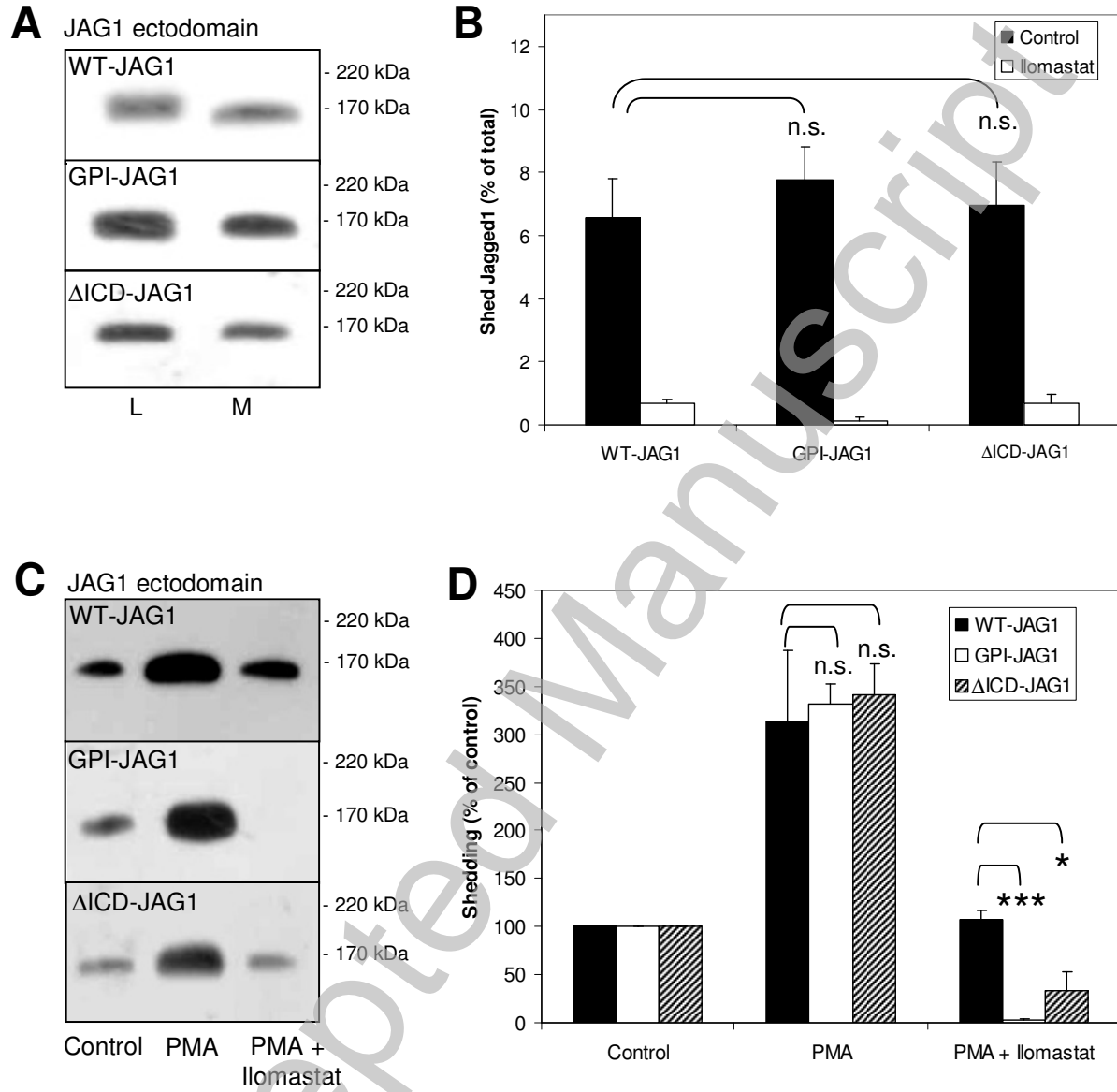
Figure 5.



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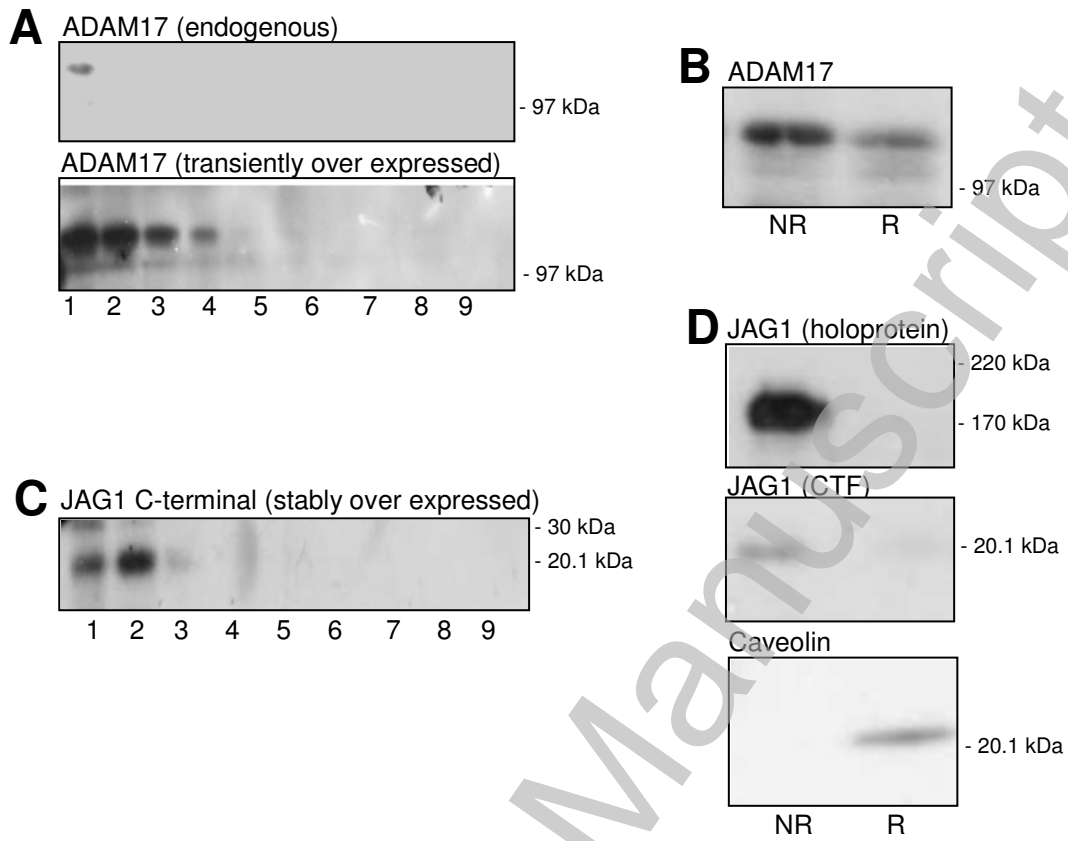
Figure 6.



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Figure 7.



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