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## **Clusterin facilitates in vivo clearance of extracellular misfolded proteins**

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### Abstract

The extracellular deposition of misfolded proteins is a characteristic of many debilitating age-related disorders. However, little is known about the specific mechanisms that act to suppress this process *in vivo*. Clusterin (CLU) is an extracellular chaperone that forms stable and soluble complexes with misfolded client proteins. Here we explore the fate of complexes formed between CLU and misfolded proteins both *in vitro* and in a living organism. We show that proteins injected into rats are cleared more rapidly from circulation when complexed with CLU as a result of their more efficient localisation to the liver and that this clearance is delayed by pre-injection with the scavenger receptor inhibitor fucoidan. The CLU-client complexes were found to bind preferentially, in a fucoidan-inhibitable manner, to human peripheral blood monocytes and isolated rat hepatocytes and in the latter cell type were internalized and targeted to lysosomes for degradation. The data suggest, therefore, that CLU plays a key role in an extracellular proteostasis system that recognises, keeps soluble, and then rapidly mediates the disposal of misfolded proteins.

### Keywords

Clusterin, facilitates, *in vivo*, clearance, extracellular, misfolded, proteins, CMMB

### Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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# Clusterin facilitates *in vivo* clearance of extracellular misfolded proteins

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**Running Title:** Clearance of extracellular misfolded proteins

## Abstract

The extracellular deposition of misfolded proteins is a characteristic of many debilitating age-related disorders. However, little is known about the specific mechanisms that act to suppress this process *in vivo*. Clusterin (CLU) is an extracellular chaperone that forms stable and soluble complexes with misfolded client proteins. Here we explore the fate of complexes formed between CLU and misfolded proteins both *in vitro* and in a living organism. We show that proteins injected into rats are cleared more rapidly from circulation when complexed with CLU as a result of their more efficient localisation to the liver and that this clearance is delayed by pre-injection with the scavenger receptor inhibitor fucoidan. The CLU-client complexes were found to bind preferentially, in a fucoidan-inhibitable manner, to human peripheral blood monocytes and isolated rat hepatocytes and in the latter cell type were internalized and targeted to lysosomes for degradation. The data suggest, therefore, that CLU plays a key role in an extracellular proteostasis system that recognises, keeps soluble, and then rapidly mediates the disposal of misfolded proteins.

**Keywords:** *clusterin, extracellular chaperone, misfolded protein, receptor-mediated endocytosis, clearance*

## Introduction

Proteins may be damaged by a variety of stresses, causing them to lose their correct fold, aggregate and form insoluble deposits[1]. These stresses can be acute, such as elevated temperature or reactive oxygen species (associated with fever and inflammation, respectively), or cumulative, low-level ongoing stresses such as hydrodynamic shear stress encountered continuously by proteins circulating in the vasculature. Inherited mutations can also destabilise protein structures and increase their propensity to aggregate. Proteostasis (**protein homeostasis**) refers to all those processes that act together to maintain the repertoire of proteins in an environment at steady state levels of abundance and function. It has been suggested that widespread failure in proteostasis is a key event in aging that promotes the onset of many of the so called protein deposition diseases that are rapidly proliferating in the aging world population[2]. In many of these diseases the location of the protein deposits is extracellular, for example in Alzheimer's disease, arthritis, type II diabetes, macular degeneration and atherosclerosis[3, 4]. While intracellular mechanisms that ensure that misfolded or damaged proteins are rapidly refolded or degraded are well characterised[5], corresponding extracellular mechanisms are largely unknown. It is known, however, that modification of human serum albumin, for example by oxidation or as a result of mutations that increase surface hydrophobicity, enhances its rate of clearance from circulating blood[6]. In addition, denatured plasma proteins have been shown to be degraded *in vivo* more rapidly than their native counterparts[7]. Collectively, these data indicate that extracellular mechanisms for maintaining proteostasis are likely to exist and act to complement the established role of intracellular chaperones and other quality control mechanisms that screen proteins in the endoplasmic reticulum prior to their secretion to the extracellular environment[4, 8].

Clusterin (CLU) was the first discovered[9] of a family of abundant extracellular chaperones (ECs) that also includes  $\alpha_2$ -macroglobulin[10], haptoglobin[11], and  $\alpha_{S1}$ - and  $\beta$ -casein[12, 13]. ECs have been shown to stabilize proteins *in vitro* and to prevent their stress-induced aggregation that can otherwise lead to the formation of amorphous or highly organised amyloid structures[3]. In the case of amorphous aggregation, CLU stabilizes a broad range of client proteins by sequestering them into soluble high molecular weight (HMW) complexes[9, 14]. It has been suggested that ECs comprise the first known elements of a novel extracellular proteostasis system, in which ECs bind to misfolded proteins *in vivo* to keep them soluble, inhibit them from forming toxic aggregates, and facilitate their bulk uptake and degradation via receptor-mediated endocytosis[8,3,4,18]. Consistent with this proposal, (i) CLU is involved in the clearance of A $\beta$  from the brains of mice[15], and (ii) insoluble protein deposits accumulate in the kidneys of aging CLU knock-out mice but not in those of age-matched wild type mice[16]. CLU has been found in the human body associated with disease-relevant insoluble protein deposits, probably as a result of its chaperone activity being overwhelmed under pathological conditions[17].

We recently demonstrated that in human plasma, major endogenous chaperone clients for CLU are fibrinogen (FGN), albumin and ceruloplasmin[18]. In the present study, FGN (340 kDa) and two model client proteins, citrate synthase (CS; 52 kDa) and glutathione-S-transferase (GST; 25 kDa) were used to study the behaviour of HMW CLU-client complexes both *in vitro* (with isolated cells) and *in vivo* (in rats). The results show that HMW CLU-client complexes are specifically bound by peripheral blood monocytes and isolated primary hepatocytes via fucoidan-inhibitable scavenger-like receptors and are internalized into lysosomes for subsequent degradation. Furthermore, in rats, rapid clearance of the complexes was fucoidan inhibitable, largely independent of the identity of the client protein, and occurred predominantly via the liver. The results provide further evidence suggesting that CLU plays a pivotal role in extracellular proteostasis.

## Materials and Methods

### Proteins

Residual soluble heated control proteins (denoted by the symbol #) and HMW complexes formed between CLU and FGN, GST or CS were formed as previously described[14].

### Isolation of peripheral human leukocytes

Human blood was obtained from a healthy donor and diluted 1:3 in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4). Blood cells were pelleted by centrifugation at 1,020 x g for 30 min, resuspended in a small volume of PBS, then diluted 1:10 in PharmLyse™ (Becton Dickinson, BD) and incubated at room temperature for 15 min. The remaining cells were pelleted by centrifugation at 700 x g for 5 min at 4°C and washed with Hank's binding buffer (HBB; 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.1% (w/v) BSA, 0.1% (w/v) glucose, pH 7.4). The success of red blood cell lysis was verified using a BD LSR II flow cytometer to acquire forward and side scatter signals; populations of lymphocytes, monocytes and granulocytes were discriminated on the basis of these same parameters. Dead cells were electronically excluded from analyses based on their staining with 1 µg/mL of propidium iodide (PI).

### Radiolabelling

Proteins and complexes for use in SPECT imaging and biodistribution studies were labelled with <sup>123</sup>I using Iodogen (Sigma-Aldrich) according to the manufacturer's instructions. A sample of radiolabelled protein was passed over a Biosep 3,000 PEEK SEC column 75 x 7.5 mm (Phenomenex) at 1 mL/min using a 2998 photodiode array detector, 600s pump and 717s autosampler (Waters) and an ACE mate™ radioactivity detector (Ortec). Profiles of the eluted

radioactivity and changes in A280 nm were obtained using the software program Empower Pro 2002, Version 5 (Waters). Purities of 95% or greater were obtained and the specific activity of the labelled proteins was typically  $89.8 \pm 8.6 \mu\text{Ci}/\mu\text{g}$  of protein ( $n = 6$ ). In the various figures and captions, the prefix  $^{123}\text{I}$  is omitted from the abbreviations of the radiolabelled proteins for simplicity.

## **SPECT imaging**

Radiolabelled proteins were formulated to a concentration of approximately  $500 \mu\text{Ci}/100 \mu\text{L}$  in PBS containing  $1 \mu\text{g}/\text{mL}$  Tween<sup>®</sup> 20. Female Sprague Dawley rats were injected via the tail vein with  $500 \mu\text{Ci}$  of the formulation. After 35 min the animals were anaesthetized using inhalant isoflurane with 200 mm/min oxygen via a nose cone fitted to the animal bed of the X-SPECT<sup>®</sup> SPECT/CT imaging system. The HRES (5" x 5") collimators were rotated to be horizontal, directly above and below the animal. Acquisitions were taken 40-60 min, 3, 6 and 24 h p.i in triplicate animals. The animals were removed from the anaesthetic and observed for recovery between each time point. After the final image was acquired, animals were sacrificed by CO<sub>2</sub> asphyxiation followed by cervical dislocation.

## **Biodistribution**

The radiolabelled proteins were formulated to a concentration of approximately  $12 \mu\text{Ci}/100 \mu\text{L}$  in PBS. Tween<sup>®</sup> 20 was added to give a concentration of  $1 \mu\text{g}/\text{mL}$  before the animals were injected via the tail vein with  $12 \mu\text{Ci}$  of the formulation. For studies investigating the effect of pre-injection with fucoidan, animals were injected with  $15 \text{ mg}/\text{kg}$  body weight of fucoidan 5 min before injection of the radiolabelled protein or complex. At designated times between 5 min and 24 h p.i., four animals were sacrificed by CO<sub>2</sub> asphyxiation followed by cervical dislocation. The animals were exsanguinated and samples of blood retained. Each organ/tissue sample was weighed and the radioactivity measured using a Wallac gamma counter 1,480 (LKB Wallac), calibrated with the radiolabelled protein solution injected into the animals. The data were corrected for radioactivity remaining in the tail, and the total blood volume was calculated according to the formula: Blood Volume (mL) =  $0.06 \times \text{BW}(\text{g}) + 0.77$ , which has a reported  $r = 0.99$  and  $p < 0.0001$  for  $n = 70$ [19].

## **Isolation of rat liver cells**

Livers were excised from Sprague Dawley rats immediately post-sacrifice. The tissue was minced using a scalpel blade and rinsed in wash buffer ( $37 \text{ mM NaCl}$ ,  $5.4 \text{ mM KCl}$ ,  $0.34 \text{ mM Na}_2\text{HPO}_4$ ,  $0.44 \text{ mM KH}_2\text{PO}_4$ ,  $0.1\%$  (w/v) BSA,  $0.5 \text{ mM EGTA}$ , pH 7.4) followed by washing in digestion buffer (wash buffer supplemented with  $0.3\%$  (w/v) BSA,  $5 \text{ mM CaCl}_2$ ,  $1 \text{ mM MgCl}_2$  and  $0.1 \text{ mg}/\text{mL}$  DNase I (Roche Diagnostics), pH 7.4). After thorough washing, the tissue was drained and incubated with  $5 \text{ mL}$  digestion buffer containing  $0.6 \text{ mg}/\text{mL}$  collagenase type 1A (Sigma-Aldrich) whilst shaking at  $37^\circ\text{C}$  for 10 min. The digestion solution was decanted through  $80 \mu\text{m}$  wire mesh, diluted in HBB and held on ice while the remaining tissue was incubated with

collagenase. A hepatocyte-enriched cell pellet was obtained by low speed centrifugation of the cell suspension for 10 min at 70 x g in a Labofuge centrifuge (Thermo Scientific). A non-parenchymal-enriched cell pellet was obtained by centrifugation at 700 x g in a Labofuge centrifuge. The separate cell pellets were washed once with HBB before being treated with PharmLyse™ (BD), according to the manufacturer's instructions. A small sample of cells was examined using an LSR II flow cytometer with PI exclusion used to estimate the viability of the cell preparations. Viability was routinely greater than 80%.

## **Flow cytometry**

Cells were placed in the wells of a 96 well microtitre plate (Greiner Bio-one) at approximately  $5 \times 10^4$  cells/well and pelleted before being incubated at approximately  $1 \times 10^6$  cells/mL in HBB on ice for 30 min with 100 µg/mL of biotinylated HMW CLU-client complex. Uncomplexed control proteins were tested individually at 100 µg/mL or co-incubated at 33 µg/mL CLU and 66 µg/mL client protein (the approximate ratio of CLU:client in the HMW complexes[14]). After washing, the cells were resuspended in HBB containing 10 µg/mL SA-Alexa Fluor®488 or Alexa Fluor®633 (Invitrogen) and again incubated on ice for 30 min. Cells were then washed twice and resuspended in HBB containing 1 µg/mL PI immediately before analysis by flow cytometry. Viable cells were gated based on PI exclusion and the data were analyzed using FlowJo7 software (TreeStar Inc.). The non-parenchymal and parenchymal fractions of the liver cells were gated based on their size and granularity. In the case of analyses of peripheral human blood leukocytes, in addition to size and granularity, CD14+ monocytes were also discriminated on the basis of green fluorescence using anti-CD14-FITC (Chemicon). The geometric mean fluorescence intensity of each sample was background corrected by subtracting the fluorescence intensity of cells incubated as above but with only SA-Alexa Fluor®488 or Alexa Fluor®633. For inhibition assays, prior to incubation with biotinylated proteins, cells were pre-incubated for 30 min on ice with 500 µg/mL fucoidan.

## **Confocal microscopy**

Hepatocytes were incubated in HBB containing 250 µg/mL SA-Alexa Fluor®488 -HMW CLU-client complexes  $\pm$  200 nM LysoTracker Red DND-99 (Invitrogen). Samples were either held on ice or incubated at 37°C for 1-2 h. The cells were examined using a Leica TCS SP system. Fluorescence and transmission images were collected using TCS NT software version 1.6.587. Gain settings were adjusted such that there was no detectable leakage of fluorescence emissions between the channels for SA-Alexa Fluor®488 and LysoTracker Red DND-99.

## **Degradation assays**

Rat hepatocytes were incubated with 250 µg/mL biotinylated HMW CLU-GST complexes  $\pm$  100 µM chloroquine in HBB at 37°C for 0-2 h. Unbound protein was removed by washing, and the cells were resuspended in reducing SDS-PAGE buffer. Protein from the cell lysates was separated

on a 12% gel and transferred to a nitrocellulose membrane. The membrane was probed using a mixture of streptavidin and biotinylated-horseradish peroxidase (2.5 µg/mL of each). After washing the membrane, enhanced chemiluminescence detection (Pierce) was performed. Biotinylated proteins resolved as < 25 kDa in mass (i.e. smaller than the smallest component of the HMW CLU-GST complex after reduction) were considered fragmented. The extent of digestion of the complexes was estimated using a GS 800 calibrated densitometer (Bio-Rad) and Quantity One software (Bio-Rad).

## Results

### Specific binding of HMW CLU-client complexes to peripheral human blood cells

It is established that CLU forms HMW complexes with misfolded proteins to inhibit their aggregation and keep them soluble[9, 14]. The fate of such complexes in the body is currently unknown. Initial cell surface binding studies using peripheral blood leukocytes found that HMW complexes formed during mild heat stress between CLU and FGN (HMW CLU-FGN[14]) bound preferentially to CD14+ monocytes (Fig. 1a;  $p \leq 0.01$ ) compared to uncomplexed control proteins. Similar results were obtained when the binding of HMW complexes formed between CLU and GST was compared to native GST (Fig. 1b;  $p \leq 0.0001$ ). Comparatively low binding by the complexes and uncomplexed control proteins to the other human blood cell types investigated (granulocytes and lymphocytes) was measured. Fucoidan, a sulfated polysaccharide ligand of many scavenger receptors, significantly inhibited the binding of HMW CLU-FGN and HMW CLU-GST to monocytes (Fig. 1c;  $p \leq 0.05$ ). Fucoidan also inhibited the lower levels of binding of uncomplexed FGN, GST and CLU (Fig. 1c;  $p \leq 0.05$ ).

### Preliminary imaging of the fate of <sup>123</sup>I-labelled HMW CLU-client complexes in rats

To investigate clearance in a mammalian system, Sprague Dawley rats were injected via the tail vein with <sup>123</sup>I-labelled HMW CLU-client complexes (formed using heat stress to partially unfold the client protein[14]), native CLU or the native client protein. In addition, to allow for the possibility that misfolded proteins generated by heating in the absence of CLU exhibit a different biodistribution in vivo, residual soluble heated proteins were also tested as controls (see Materials and Methods). Preliminary SPECT imaging indicated that, within 45 min post injection (p.i.), native control proteins, residual soluble heated control proteins and HMW CLU-client complexes were all localized to the upper abdomen in the general region of the liver, spleen and nearby organs (Fig. 2a). The overwhelming majority of the radioactivity remained localized in the upper abdomen for the entire 24 h of the experiment, although radioactivity in the thyroid gland was seen

to increase during this period (Supplementary Fig. 1a). At 1 h p.i., the proportion of the total injected dose of radiation remaining in the blood was significantly less for HMW CLU-GST and HMW CLU-FGN complexes, compared to the control proteins (Fig. 2a;  $p \leq 0.01$ ). Using the weight of the animals to approximate their total blood volume, it is evident that at this time only about 5% (or less) of the  $^{123}\text{I}$ -radioactivity originally associated with the HMW CLU-client complexes was present in the blood. Similarly rapid clearance of proteins injected into rats has been reported before[6].

## Biodistribution studies

A more detailed and comprehensive biodistribution study was next carried out, including starting the measurements at earlier time-points following the injection of the radiolabelled proteins (Fig. 2a). In these experiments using HMW CLU-FGN and HMW CLU-CS, native proteins were used as controls because (i) previous work showed that HMW CLU-FGN retains the overall secondary structure content of its native components and HMW CLU-CS is no more similar in terms of secondary structure content to its residual heated components than its native components[14], and (ii) preliminary SPECT imaging indicated that biodistribution was similar regardless of the nature of the control chosen (see above). In light of the results showing fucoidan-inhibition of the binding of HMW CLU-client complexes to CD14+ monocytes (above), biodistribution experiments were repeated comparing rats pre-injected with 15 mg/kg of fucoidan with those that were not. At 5 min p.i., when the contribution of any metabolism to the level of radioactivity in the blood would be minimal, the extent of clearance of  $^{123}\text{I}$ -HMW CLU-client complexes was significantly greater than that of the relevant control proteins ( $p \leq 0.01$ ), and represented approximately 75% of the complexes being cleared within this time (Fig. 2c). In the case of animals injected with  $^{123}\text{I}$ -FGN, however, by 15-30 min p.i. the level of radioactivity present in the blood was similar to that of animals injected with  $^{123}\text{I}$ -HMW CLU-FGN. It is feasible that at these longer time points, the level of radioactivity in the blood of the latter animals was supplemented with free  $^{123}\text{I}$  and  $^{123}\text{I}$ -tyrosine derivatives released back into the bloodstream after intracellular processing of the radiolabelled proteins. This possibility is supported by a time-dependent increase in the radioactivity located in the thyroid gland (Supplementary Fig. 1b). Thus, the data clearly show that the *in vivo* clearance of client proteins is significantly enhanced when they are complexed with CLU. At 5 min p.i., fucoidan pre-treatment in all cases retarded the clearance of the radiolabelled proteins from the bloodstream (Fig. 2c). This effect was most significant for animals injected with  $^{123}\text{I}$ -HMW CLU-FGN ( $p \leq 0.01$ ), where fucoidan produced close to a 4-fold increase in the amount of radioactivity present in the blood at this time point; the corresponding increase was approximately 3-fold for  $^{123}\text{I}$ -HMW CLU-CS and  $^{123}\text{I}$ -FGN and closer to 2-fold for  $^{123}\text{I}$ -CLU and  $^{123}\text{I}$ -CS.

In the rat body, for all labelled proteins, at 5 min p.i. radioactivity was most concentrated in the liver (Fig. 3). However, animals injected with  $^{123}\text{I}$ -FGN also had a high concentration of radioactivity in the kidneys. The apparent rapid uptake of  $^{123}\text{I}$ -FGN into the kidneys may explain the increased clearance of FGN compared to the other uncomplexed control proteins. The spleen

appears to be a secondary site for the uptake of HMW CLU-client complexes. However, considering the mass of the rat liver is approximately 19 times greater than the mass of the spleen (Wyatt, personal observation), the contribution of the latter to the clearance of the labelled proteins was proportionally much less. Pre-injection with fucoidan changed the biodistribution profile in all cases (Fig. 3). In particular, uptake by the liver was significantly reduced ( $p < 0.01$  in all cases). Other statistically significant changes included a reduction in uptake of  $^{123}\text{I}$ -CS and  $^{123}\text{I}$ -CLU by the spleen and increased uptake of  $^{123}\text{I}$ -FGN,  $^{123}\text{I}$ -CS and  $^{123}\text{I}$ -CLU by the lung ( $p < 0.01$  in all cases).

### **Specific binding of HMW CLU-client complexes to isolated rat hepatocytes**

To gain further insight into the interaction of HMW CLU-client complexes with cells found in the liver, experiments were carried out on freshly isolated rat liver cells. In these experiments HMW CLU-client complexes were found to bind to isolated rat hepatocytes to a much greater extent compared to the non-parenchymal cells (Supplementary Fig. 2). When hepatocytes were incubated with HMW CLU-client complexes, or the relevant control proteins at the same final mass concentrations as in the complex (i.e. molar ratio of CLU:client of 1:2[14]), 4-5 fold greater binding was measured for the complexes compared to the control proteins (Fig. 4a;  $p \leq 0.01$ ). When hepatocytes were treated with fucoidan, the binding of biotinylated HMW CLU-FGN and HMW CLU-GST was reduced by 50-75% (Fig. 4b;  $p \leq 0.01$ ). Fucoidan was also found to inhibit about 50% of the binding of residual soluble heat-treated biotinylated FGN and GST ( $p \leq 0.01$ ), although there was no statistically significant effect on the binding of native FGN, native GST, native CLU or heat-treated CLU. Taken together, the data shows that hepatocytes preferentially bind HMW CLU-client complexes compared to uncomplexed control proteins and this preferential binding occurs at least in part via scavenger-like receptors.

To pursue this latter point further, the binding of HMW CLU-client complexes to the surface of freshly isolated rat hepatocytes was examined by confocal microscopy (Fig. 5a). When the hepatocytes were incubated at physiological temperature (2 h at  $37^\circ\text{C}$ ) the images show that binding of the complexes to the cell surface was followed by their internalization. The internalized HMW CLU-client complexes were co-localised with Lysotracker Red DND-99 labelled vesicles (Fig. 5b), indicating that the complexes had been transported into acidic compartments. Very similar results were obtained using CLU-GST complexes (data not shown). The ability of hepatocytes to degrade extracellular CLU-client complexes was then investigated by using Western blots to follow the proteolytic breakdown of biotinylated complexes. Treatment with chloroquine, an inhibitor of lysosomal proteases, reduced by about 50% the proteolytic fragmentation of biotinylated complexes incubated with rat hepatocytes for 2 h at  $37^\circ\text{C}$  ( $p \leq 0.01$ ; Fig. 5c). These findings are consistent with hepatocytes endocytosing and trafficking complexes to lysosomes where they are degraded.

## Discussion

Previous work has shown that CLU is a potent EC with the ability to bind to a broad range of client proteins, and suggested that it is part of a larger system of extracellular proteostasis in which ECs direct misfolded extracellular proteins to cell surface receptors for uptake and degradation[4]. CLU and CLU-A $\beta$  complexes are known to bind to the LDL family receptor megalin (LRP2;[20, 21]). However, in humans the distribution of megalin is highly restricted; the most prominent expression is in the proximal tubules of the kidney but it is also expressed in the choroid plexus epithelium and ependymal cells lining the brain ventricles[22]. Owing to this restricted expression, we reasoned that this receptor was unlikely to function as a bulk systemic pathway for binding and disposal of HMW CLU-client complexes, although it may play a critical role in some circumstances (e.g. in clearance of CLU-A $\beta$  in the brain;[20]). Indeed, we were unable to detect significant binding of HMW CLU-FGN or HMW CLU-CS complexes to immobilized megalin in surface plasmon resonance analyses, although binding of CLU to megalin was detected (Wyatt et al., unpublished).

In contrast, scavenger receptors are widely expressed by many tissues in the body, including the liver and cells of the reticuloendothelial system (e.g. monocytes)[23]. Scavenger receptors are known to bind preferentially to proteins that have been damaged by, for example, reactive oxygen species or advanced glycation, as well as proteins treated with chemical denaturants[24, 25]. Furthermore, the binding of misfolded proteins by scavenger receptors on platelets induces their activation[26]. We examined the binding of HMW CLU-client complexes to peripheral blood leukocytes and found that they bound preferentially to monocytes compared to uncomplexed control proteins and that this binding was significantly inhibited by fucoidan (which binds to many scavenger receptors[27]; Fig. 2). Monocytes express a number of scavenger receptors that bind fucoidan[28, 29] – thus, these receptors are prime candidates for the binding of HMW CLU-client complexes. While fucoidan is known to bind to class A[30], E[31] and F[32] scavenger receptors, like most sulphated polysaccharides, it also binds to many other proteins including other kinds of receptors[27]. Therefore, the results suggest that HMW CLU-client complexes bind to scavenger-like receptors on monocytes and other cell types, however, the identity of the specific receptor(s) involved remain to be established.

Preliminary SPECT imaging experiments indicated that HMW CLU-client protein complexes were rapidly cleared from the blood, and by 1 h p.i. were overwhelmingly localised into a position in the upper abdomen (Fig. 2a). In the subsequent biodistribution experiments, the level of radioactivity present in the blood after injection of labelled proteins or complexes was influenced by (i) clearance of labelled species from the blood (which will act to decrease the level of radioactivity present), and (ii) cellular uptake, metabolism, and release of free  $^{123}\text{I}$  or  $^{123}\text{I}$ -tyrosine derivatives back into the blood (which will be more prominent with time and act to increase the

radioactivity in the blood). Importantly, regardless of these caveats, the data in Fig. 2b show that complexation with CLU significantly increased the extent of clearance of client proteins from the bloodstream at 1 h p.i. compared to that of uncomplexed control proteins. This was also true at 5 min p.i. (Fig. 2c). It is however important to realise that the magnitude of these differences in clearance rates are not of primary importance in the physiological context. The critical point is that in the absence of clusterin (or some other extracellular chaperone) misfolding extracellular proteins will aggregate and precipitate, and no longer be available as soluble species for clearance via receptor-mediated endocytosis. Instead, the body will be faced with an insoluble deposit which may be much more difficult to clear. The results presented further indicate that CLU-client protein complexes are rapidly cleared *in vivo* from the circulation by a fucoidan-sensitive mechanism, consistent with the involvement of scavenger-like receptors. It was also shown that fucoidan significantly retarded the clearance of the uncomplexed control proteins from the bloodstream (Fig. 2c). Whether or not the clearance mechanisms implicated here are the same as those already described for damaged or modified ligands such as oxidized LDL, modified albumin and advanced glycation end products remains to be confirmed. The interaction of fucoidan with cell surface receptors other than scavenger receptors may provide an explanation for its partial inhibition of the uptake of some uncomplexed control proteins. Fucoidan is known to bind to L-selectin and can thereby interfere with leukocyte rolling[33, 34]. In addition, fucoidan inhibits the phagocytosis of apoptotic leukocytes by endothelial cells, even though it has been shown that the latter do not express fucoidan-inhibitable scavenger receptors[35]. Nevertheless, scavenger receptors bind a diverse array of polyanionic ligands and therefore specific binding of CLU, CS or FGN (all negatively charged at physiological pH) to scavenger receptors is also possible[36].

We have previously shown that clusterin is able to form HMW CLU-client complexes with a broad range of proteins *in vitro* [37, 38] and that regardless of the specific client protein, the complexes have very similar physical characteristics [39]. All of the experiments described here were carried out using the endogenous client protein fibrinogen [40]. We have supplemented these results with those of experiments using recombinant (protozoan) GST (a well established model protein for studying the chaperone activity of clusterin at elevated temperature [37]) and CS (an intracellular enzyme, which can be released by dead or dying cells, that forms complexes with clusterin at physiologically relevant temperatures [41]) to demonstrate that the clearance mechanism involved is almost certainly a general one and is not specific to individual client proteins. The combined results from SPECT imaging and biodistribution studies indicated that the liver was the major organ involved in clearance of radiolabelled HMW CLU-client complexes from the blood (Fig. 2a & 3). The spleen appears to be a secondary site of clearance. In addition, accumulation of FGN and CLU in the kidney was also apparent (Fig. 3). Although the underlying reasons are unclear, similar uptake of blood-borne human FGN into the liver and kidney of rats has previously been reported[42]. The uptake of CLU into the kidney is likely to be due to the endocytic receptor known as megalin[20, 43]. Pre-injection of fucoidan significantly retarded uptake of radiolabelled HMW CLU-client complexes and proteins by the liver; this effect was greatest for HMW CLU-FGN complexes (Fig. 3,  $p < 0.01$  in all cases). These differences were

apparent even though fucoidan increased the proportion of radiolabelled proteins remaining in the blood at this time (Fig. 2c and data not shown), which owing to the blood sinuses within the liver tissue, would have had the effect of partially masking the decreased uptake by liver cells. The fucoidan-induced increase in localization in the lung could be accounted for by the greater fraction of the injected dose remaining in the blood under these conditions; the lungs are very highly vascularized, and it has been estimated that more than 15% of the total blood volume of a rat may be held in the (non-perfused) lungs[44]. Other tissues and organs such as muscle, skin, the brain and gut showed very limited uptake of complexes (Fig. 3). We have previously shown that clusterin-client protein complexes are very large (50-100 nm in diameter, approaching the size of viruses)[39] and thus are unlikely to enter tissues or organs without the aid of specific transport system(s). Our previous work strongly suggests that these large complexes are vehicles for the safe disposal of misfolded extracellular proteins[3, 4, 8, 39]. Therefore, the current results suggest that those tissues and organs for which only limited uptake of complexes was measured lack relevant transport systems and are not primary sites for the *in vivo* disposal of misfolded proteins complexed with clusterin.

Given the apparent major role of the liver in clearance of HMW CLU-client complexes, we next examined interactions between HMW CLU-client complexes and isolated rat hepatocytes. Using electronic gating of cell populations based on forward and side laser scatter signals, complexes showed substantially greater binding to the larger hepatocytes than smaller non-parenchymal cells (the latter includes endothelial and Kupffer cells; Supplementary Fig. 2). Liver cells are known to express many receptors with important roles in targeting plasma proteins for clearance. This includes certain members of the LDL receptor superfamily and several scavenger receptors, which are all capable of binding and facilitating the subsequent degradation of plasma proteins. The extent of binding of HMW CLU-client complexes to isolated hepatocytes was 4-5 fold greater than that of protein controls and was significantly inhibited by fucoidan (Fig. 4). Fucoidan also significantly inhibited the binding of heat-treated (but soluble) FGN and GST but had lesser effects on the binding of native FGN and GST, and CLU (heated or not). These results are all consistent with rat hepatocytes expressing one or more types of scavenger-like receptors which preferentially bind to misfolded proteins, including those carried “in bulk” in HMW CLU-client complexes. Although hepatocytes were once thought to only express class B scavenger receptors (which are insensitive to fucoidan;[45]), recent work suggests that they express one or more other types of scavenger receptors sensitive to inhibition with polyanions[46]. Lastly, confocal microscopic and Western blotting analyses showed that once bound by rat hepatocytes, over the course of a few hours, HMW CLU-client complexes were internalized into acidic compartments and proteolytically degraded by lysosomal proteases (Fig. 5). Previous work has shown that clusterin and clusterin-A $\beta$  complexes (specifically) can be cleared by receptor-mediated endocytosis, via the cell surface receptor megalin, and are degraded in lysosomes [47]. A number of other studies have also suggested that clusterin and clusterin-ligand complexes can be cleared by the same route [48, 49]. Data presented here also strongly implicates the classical route of

receptor-mediated endocytosis and lysosomal degradation in the clearance of clusterin-client protein complexes. We cannot exclude the possibility that other processes (e.g. autophagy) might be involved in the intracellular disposal of clusterin-client complexes, although there is currently no evidence to support this.

Many biological functions have been proposed for CLU including roles in the clearance of cellular debris[43], leptin[50] and amyloid  $\beta$  peptides[20]. It is likely that the ability of CLU to interact with such a broad range of structurally diverse ligands is the result of the propensity of this chaperone to bind to areas of exposed hydrophobicity. Taken together with our previously published work[21,8,4], the results described here suggest that CLU plays a specific role in recognising misfolded extracellular proteins, maintaining them in a stable and soluble state, and then facilitating their bulk clearance and subsequent degradation via receptor-mediated endocytosis. The data from experiments where fucoidan was used as a pre-treatment further indicate that CLU-client complexes are preferentially recognized by scavenger-like cell surface receptor(s), especially those present in the liver. Thus, although we have not identified the specific receptors involved, results obtained from both *in vitro* and *in vivo* systems suggest that scavenger-like receptors are likely to recognise the misfolded protein “cargo” carried by CLU and mediate its rapid clearance from extracellular spaces in the body.

Misfolded proteins have a tendency to self-assemble to form insoluble deposits, such as those observed in protein deposition diseases[1]. As many of these diseases are associated with extracellular protein deposits, the high levels of CLU and other ECs in extracellular fluids, and their ability to form complexes with client proteins under physiological conditions, suggests that they play a key role in the prevention of protein deposition diseases. While low concentrations of misfolded extracellular proteins might be recognised locally at the plasma membrane and internalized by scavenger-like receptors (Fig. 6a), the ability of circulating ECs to sequester misfolded proteins into stable and soluble complexes is likely to be an important initial step in their transport to endocytic receptors for disposal (Fig. 6b). In addition, it has been reported that  $\beta$ -sheet-rich amyloid fibrils and denatured protein aggregates are able to stimulate tissue type plasminogen activator-mediated plasminogen activation[51, 52]. Such stimulation may constitute an additional system, which could function in tandem with ECs and act to maintain the existing pool of extracellular proteins in their correct fold (Fig. 6c).

It has become increasingly clear that control of the multiple conformational states of proteins and the maintenance of proteostasis in general is a vital life process[2, 53], although the mechanism by which such regulation occurs in the extracellular environment has previously proved elusive. The present study, however, provides direct evidence that CLU performs a critical *in vivo* role by binding and then internalising misfolded proteins located in the extracellular environment for subsequent degradation. It is likely that this effect is part of a larger system for the control of

protein misfolding and aggregation in extracellular environments. In addition, it opens the door for the discovery of specific receptors for CLU-client complexes and the elucidation of how other known ECs synergise with each other and with extracellular protease systems. The current work provides a shift in paradigm for our detailed understanding of the processes that impact upon the occurrence and potential treatment of many of the highly debilitating and frequently fatal protein deposition disorders that are rapidly becoming the most feared and costly diseases in much of the modern world.

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## Figure Legends

**Fig. 1** Binding of HMW CLU-client complexes and control proteins to human peripheral blood leukocytes and the effect of fucoidan pre-treatment, assessed by flow cytometry. Freshly isolated human leukocytes were incubated with 100  $\mu\text{g}/\text{mL}$  biotinylated (A) clusterin (CLU), fibrinogen (FGN) or HMW CLU-FGN, or (B) GST or HMW CLU-GST, and then 5  $\mu\text{g}/\text{mL}$  streptavidin (SA)-Alexa Fluor® 633. In separate experiments the binding of the same proteins to CD14+ monocytes pre-incubated with or without 500  $\mu\text{g}/\text{mL}$  fucoidan was measured as described (C). The results are the geometric mean of the Alexa Fluor® 633 fluorescence in arbitrary fluorescence units (AFU) ( $n = 3 \pm$  standard deviation). + Denotes significantly higher binding of HMW CLU-client complexes compared across the cell types and also compared to the binding of uncomplexed control proteins to all cell types (as shown on the same panel; Tukey HSD, and Student's t-test,  $p < 0.01$ ). \* Denotes significant inhibition by fucoidan (Student's t-test,  $p < 0.05$ ).

**Fig. 2** Clearance of blood-borne  $^{123}\text{I}$ -labelled HMW CLU-client complexes and control proteins in Sprague Dawley rats. (A) SPECT imaging of the lower body of a rat (orientation indicated by the labels Abdomen and Tail) injected with  $^{123}\text{I}$ -HMW CLU-FGN via the tail vein. Progressively higher levels of radioactivity are indicated by the pseudocolour gradient moving from blue to green, then yellow, red, and finally white. The times shown are p.i. and the images shown are representative of three different experiments. Similar results were obtained for native and residual soluble heated CLU and FGN (not shown). (B) Rats were injected with  $^{123}\text{I}$ -labelled HMW CLU-client complex or native control proteins. The radioactivity per gram of blood was measured 1 h p.i. and the total activity was calculated by estimating the blood volume of each rat (see Materials and Methods). Data points represent means ( $n = 3 \pm$  standard deviation) (C) Panels showing the levels of circulating radioactivity in blood up to 30 min after rats were injected with  $^{123}\text{I}$ -labelled HMW CLU-client complex or uncomplexed control protein with or without pre-injection with fucoidan. Data points represent means ( $n = 4 \pm$  standard deviation). + Denotes significantly higher radioactivity in the blood of rats pre-injected with fucoidan (Student's t-test,  $p < 0.01$ ). \* Denotes significantly lower radioactivity compared to CLU and the relevant uncomplexed control proteins (Tukey HSD,  $p < 0.01$ ).

**Fig. 3** Biodistribution at 5 min post-injection of  $^{123}\text{I}$  labelled HMW CLU-client protein complexes and control proteins in Sprague Dawley rats, with and without pre-injection of fucoidan. The identity of the corresponding HMW CLU-client complexes and control proteins are indicated on each panel. Data points represent the mean percentage of the injected dose/g of tissue ( $n = 4 \pm$  standard deviation) and are corrected for any radioactivity remaining in the tail. \*Denotes significantly reduced radioactivity in animals pre-injected with fucoidan and + denotes significantly increased radioactivity in animals pre-injected with fucoidan (Student's t-test,  $p < 0.01$ ).

**Fig. 4** Flow cytometric measurements of the binding of HMW CLU-client complexes and control proteins to rat liver. (A) Hepatocytes were incubated with biotinylated HMW CLU-FGN or HMW CLU-GST, or mixtures of (i) biotinylated CLU and biotinylated FGN, or (ii) biotinylated CLU and biotinylated GST (at the same final mass concentrations, and with CLU:client mass ratio = 1:2 in both cases), and then incubated with streptavidin (SA)-Alexa Fluor® 488. \* Indicates significantly less binding of the uncomplexed proteins compared to that of the HMW CLU-client protein complexes (Student's t-test,  $p < 0.01$ ). (B) In separate experiments, hepatocytes were incubated with or without fucoidan before incubation with biotinylated (i) FGN, residual soluble heated FGN (FGN<sup>#</sup>) or HMW CLU-FGN, or (ii) GST, residual soluble heated GST (GST<sup>#</sup>) or HMW CLU-GST, or (iii) CLU or residual soluble heated CLU (CLU<sup>#</sup>), followed by SA-Alexa Fluor® 488. + Indicates significantly less binding of the uncomplexed proteins compared to the HMW CLU-client complexes (Student's t-test,  $p < 0.01$ ). In all cases, the results are the geometric mean fluorescence in arbitrary units (AU;  $n = 3 \pm$  standard error).

**Fig. 5** Binding, internalization and degradation of HMW CLU-client complexes by rat hepatocytes. (A) Confocal fluorescence image and the corresponding transmission image of rat hepatocytes incubated with Alexa Fluor® 488-labelled HMW CLU-FGN for 2 h on ice. (B) Confocal fluorescence images of hepatocytes incubated with Alexa Fluor® 488-labelled HMW CLU-FGN and LysoTracker Red DND-99 for 2 h at 37°C. Also shown are an overlay of the two fluorescence images and the corresponding transmission image (as indicated on the figure). The scale bars are 10  $\mu$ m. (C) Effect of the lysosomal protease inhibitor chloroquine on the degradation of internalized CLU-GST complexes by rat hepatocytes (see Materials and Methods). \*Denotes significant inhibition of fragmentation by chloroquine (Student's t-test,  $p < 0.01$ ).

**Fig. 6** Proposed mechanism for maintenance of extracellular proteostasis. Under normal physiological conditions (A) scavenger receptors may directly bind misfolded proteins locally. (B) Circulating extracellular chaperones target and bind to misfolded proteins, maintaining their solubility and facilitating their transport to scavenger receptors. (C) When extracellular proteostasis is disrupted, insoluble protein aggregates can form giving rise to activated proteases (e.g. plasmin). Extracellular chaperones interact with the proteolytic fragments and facilitate their transport to scavenger receptors. In all cases, delivery to scavenger receptors results in the intracellular transport of misfolded proteins to lysosomes for degradation.