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Unfolded Protein Response-Induced ERdj3 Secretion Links ER Stress to Extracellular Proteostasis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Anne Nielsen

1st Editorial Decision 11 June 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all three referees highlight the impact and quality of your findings and they would consequently support publication of your manuscript following appropriate revision. The reports are overlapping in some points, but I would like to mention that while data supporting a correlation between ERdj3 secretion and amyloid deposits in patient material would clearly strengthen the medical implications of the study, this point is not an absolute requirement from our side.

I would ask you to focus your efforts on the following points:

- Please extend the literature discussion to more clearly place ERdj3 in the context of extracellular chaperones (ref #1)

-> Please also add further data to address the interplay between ERdj3 and BiP in secretion and association with misfolded client (ref#2)

-> Along the same lines, we would also ask you to follow the recommendations by ref#3 to look more closely at the disaggregation activity of secreted ERdj3 and whether this activity would occur during the physiological stress response (also mirroring point 1 raised by ref #2)

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS:

Referee #1:

In this report, Genereux and co-workers demonstrate the secretion of ERdj3 to the extracellular space, through an UPR-dependent mechanism. ERjd3 is proposed to have a function on protein folding in the extracellular space. This observation is highly relevant in order to understand possible toxic and protective mechanisms related to protein misfolded disorders. The authors claim that this is the first report indicating a cellular response to misfolded protein in the extracellular space.

General Comments:

This paper is well-written, it represents a very clear story and the overall structure of the manuscript is well organized. The phenotypes that authors show are robust and convincing. Also, the conclusions of all experiment are fair in their interpretations. This manuscript could be accepted after minor revisions.

In order to improve the impact of this manuscript, I think it may be interesting to perform some experiment supporting the principal concept, and highlight the biomedical application. If possible, a correlation between ERdj3 expression in and amyloid beta deposition could be provided using brain tissue derived from AD or JCD patients.

The authors should discuss other papers showing secretion of ER foldases. The way in which the study is presented gives the impression that this is the first chaperone described to be secreted, but this is not the case. PDI and ERp57 have been shown to be decreased and even participate in the extracellular folding of proteins and there are even reviews on the subject (see Antioxid Redox Signal. 2006 Mar-Apr;8(3-4):312-24.). BiP has been also shown to express at the extracellular membrane, and has been used for cancer immunotherapy. Finally, many papers from Guido Kroemer¥s lab showed that calreticulin (together with ERp57) are expressed in the plasma membrane on an ER stress-dependent manner, and this concept has been also exploited for cancer immunotherapy. ERp57 has been shown to associate with amyloid beta in CSF of patients also. Finally, one of the coauthors paper (R Kaufman) initially reported the secretion of many ER foldases upon ER stress (Dorner et al 1990, JCB)

A non-functional ERdj3 (instead ERdj3-KDEL) could be used in experiment related with aggregation of Abeta (Figure 3) or the PrP toxicity (Figure 4). ERp57 has been shown to have functions independent of the enzymatic activity.

Referee #2:

The manuscript submitted to EMBO J. by Wiseman and colleagues reports on the extracellular activity of a chaperone, ERdj3, which normally resides in the ER lumen. The study was initiated by a search for ER lumenal chaperones that might be secreted and then retain activity extracellularly, and the prime candidate was ERdj3. ERdj3 is a known BiP cochaperone, contributing to ER protein folding and degradation and is a UPR target. By using cell lines engineered in this laboratory in which the ATF6 and XBP1 legs of the UPR can be selectively activated in a stress-independent mechanism it was found that only ATF6 induction liberated ERdj3 from the ER, even though the mRNA encoding ERdj3 was induced in both cases. The mechanism underlying this observation lies in the fact that XBP1 induction also triggers ERdj3 degradation. To show that extracellular ERdj3 was-as predicted-functioning as a chaperone, data were obtained indicating that A(beta) aggregation was attenuated in vitro by ERdj3, that ERdj3 bound resin conjugated with an unfolded (but not folded) polypeptide, that conditioned and concentrated media from ERdj3 overexpressing cells tempered TPrP-dependent vacuolization in a neuroblastoma cell model, and that ERdj3 associated with APP fragments when coexpressed. A clever experiment was then performed to assess whether ERdj3 associates with unfolded polypeptides in the ER (and they ferries them to the extracellular space) or whether association occurs extracellularly. By using a series of tools pioneered in the Kelly lab, and by examining conditions of co-expression versus co-incubation, it was clear that interaction occurs within the cell. Further, ERdj3 preferentially recognized a folding defective protein (TTR A25T) but not wild type TTR in several cell models. Perhaps the most striking experiment details the use of Tafamidis in augmenting TTR A25T secretion while decreasing ERdj3 association, as expected based on the author's model. Interestingly, ERdj3 was unable to aid the secretion of an unstable, ER-retained TTR variant (D18G), which is in contrast to the effects of another secreted chaperone-like molecule, clusterin. Finally, data were presented that ATF6 activation decreased ERdj3-TTR interaction, possibly in a BiP-dependent manner (since the H53Q mutant negated this effect). Consistent with this hypothesis, introduction of a BiP over-expression vector similarly decreased the degree of interaction.

Collectively, this an impressive body of work but direct proof of whether a specific substrate ferried by ERdj3 is prevented from aggregating when bound to ERdj3 extracellularly is not shown. While an aberrant polypeptide, e.g., TTR A25T, is clearly a substrate, does the chaperone prevent the toxic effects of this molecule once secreted? The other experiments, noted above, in which ERDj3 prevented aggregation and/or toxicity used other substrates. Therefore, the authors should be cautious about their conclusions that ERdj3 association in the cell and then secretion "protects" the extracellular compartment.

A second point concerns the question of whether ERdj3 is acting alone or in a complex with other chaperones. The effect of conditioned media, as used for the TTR studies, fails to differentiate between these scenarios. However, BiP is not secreted upon ATF6 activation (Fig. 2A), although a BiP antibody (instead of the anti-KDEL antibody) should be used to confirmed this observation. Interestingly, BiP is secreted from yeast exposed to UPR inducing conditions (Barlowe and colleagues, Mol. Biol. Cell, 2001; Miller and colleagues, Genetics 2009); therefore, the last sentence of the abstract needs to be slightly modified and this fact should be mentioned. Is there any evidence of extracellular BiP when the TTR-ERdj3 complex was observed extracellularly? In addition, data suggesting that BiP affects ERdj3-TTR interaction is indirect: Fig. 6 seems to lack data indicating that the levels of BiP rise. More definitive results might be obtained if there is an effect when BiP is silenced, perhaps by shRNA or subtilase toxin administration. Addressing some of these minor points would significantly strengthen the authors' conclusions.

Regardless of these concerns, this manuscript reports on a new and important phenomenon in an active (and medically relevant) area of research. The quality of the data is excellent and the myriad tools and systems used by the investigators is a notable strength of the manuscript. The paper will be an excellent addition to the journal.

Other points:

1. p. 5, last full paragraph, it is confusing that HEK293T cells are again listed amongst the other cell lines (since the cells were already discussed and used). This is especially relevant since the signal is so much weaker in Fig. S1C compared to Fig. 1A.

2. The time point used to obtain the data in Fig. S2A is not given. Also, is BiP secreted if longer ATF6 (or XBP1) induction times are used (viz. Fig, 2A)?

3. As reported in Zhou et al., 2012, the vacuolization study could be buttressed by the use of another reporter for TPrP (toxic) effects in the neuroblastoma cell line.

4. In Fig. 5B, why is the level of TTR A25T in the IB so weak when probing with anti-FLAG/M2? This is not the case in other experiments when co-expression is being analyzed (compare for example Fig. 5B and C).

5. p. 12, "significantly" should be deleted since the effect of Tg pretreatment changes the relative recovery of ERdj3 only modestly (but yes, the data are statistically significant...)

6. Fig. 2C and E. Although p values are given in the legend, I failed to note asterisks in the figures, which are supposed to reflect the provided statistics.

7. HYOU1 is not a commonly known chaperone so additional information on its function should be provided.

8. Fig. 3D. The administration of BSA yields an effect that is intermediate between the buffer control and ERdj3. Quantitative data need to be provided for replicates of this experiment, or another control should be used.

9. Fig. S2. The arrow for Grp94 does not indicate a specific protein species.

Referee #3:

Genereux et al show that ERdj3, an ER-resident BiP co-chaperone, can be secreted from cells upon ER stress both from cells in vitro and in vivo. This secretion is specific for ERdj3 (Bip and HYOU1, 2 other UPR induced chaperones are not secreted), is dependent on the AFF6 and not the Xbp-1

branch of the UPR, and occurs via classical ER secretion pathways (KDEL dependent, Brefeldin A sensitive). ERdj3 is found to be co-secreted with misfolded ER clients (e.g. mutant TTR) the extent of which being dependent on a functional interaction of ERdj3 with BiP. Recombinant ERdj3 was found to have strong anti-aggregation power against e.g. Abeta (1-40) peptides and media derived from ERdj3 over expressing cells associated with misfolded proteins and reduced toxicity of extracellular aggregates.

Overall, I find this a highly interesting data set and the paper is well-written.
In my view, the data are fully conclusive to show ERdj3 co-secretion with unfolded clients and suggest that this indeed may be a backup mechanism under conditions of BiP shortage (and thus impaired ERAD) in order to dump misfolded clients into the extracellular milieu. Regarding the suggested other role of ERdj3 in regulating extracellular proteostasis, I have some questions that remain to be solved.

1. The finding that recombinant ERdj3 has strong anti-aggregation power against e.g. Abeta (1-40) is exciting in itself and of course leads to the suggestion that secreted ERdj3 may have protective effects in Alzheimer's disease. It would first of all have been nice(r) to show that also medium from ERdj3 over expressing cells would protect against toxicity from Abeta peptides rather than from toxicity of PrP. Alternatively, the cellular data with PrP could have been complemented with effects of ERdj3 on in vitro PrP aggregation (rather than Abeta).

2. The cellular data on PrP aggregation being attenuated by media from ERdj3 over expressing cells requires a number of additional controls. To proof that it is mediated by excreted (free) ERdj3, anti-body blocking experiments are required. In addition, recombinant ERdj3 could be used as positive control and media collected from ERdj3-KDEL over expressing cells as additional negative control.

3. I wonder what the evidence is that ERdj3 can leave the ER without clients such that it is available for extracellular chaperoning. On one hand, the authors state that client release may not happen extracellularly because BiP is not out there (I think this is a valid argumentation). On the other hand, conditions that induce ERdj3 excretion are usually such that they will involve co-excreting with clients, meaning no free chaperone will be present in the extracellular milieu. I wonder whether this actually explains why only co-expression and not co-incubation results in IP-interaction between mutant TTR and ERdj3 (figure 5? I realise that the data with the conditioned medium in figure 4 are suggesting otherwise, but one may argue that the preparations of the conditioned medium in these experiments artificially induces the release of clients from ERdj3. Is there any manner by which the authors can exclude this possibility?

4. Figure 3D: I am somewhat disturbed by the strong effect of BSA in this panel: can the authors explain this (minor comment)

1st Revision - authors' response 09 September 2014

OUR RESPONSE TO THE EDITOR AND REVIEWER COMMENTS

REVIEWER COMMENTS HIGHLIGHTED BY THE EDITOR

EDITOR COMMENT #1. "*Please extend the literature discussion to more clearly place ERdj3 in the context of extracellular chaperones*."

OUR RESPONSE TO EDITORIAL COMMENT #1. As suggested by the editor and Reviewer #1, we include additional discussion on previous work describing the secretion of ER chaperones and proteostasis factors (please see **Our Response to Reviewer #1 Comments #1 & 2** for additional discussion). We also now discuss our observations showing UPR-dependent increases of ERdj3 secretion in the context of this previous work, specifically highlighting similarities and differences supported by the work described in our manuscript. We specifically highlight the distinction between the secretion of a large fraction of newly-synthesized ERdj3 relative to the small fractions of newly-synthesized BiP and calreticulin trafficked to the cell membrane, as previously reported. The changed text in the revised manuscript, is found directly below.

> Page 3, Paragraph 2:" *Extracellular proteostasis capacity is regulated by secreted proteins that prevent the formation of protein aggregates associated with disease. The best characterized secreted chaperones such as clusterin directly bind misfolded proteins in the extracellular environment and prevent their aggregation through an ATP-independent 'holdase' mechanism (Wyatt et al, 2012). Deletion of clusterin predisposes mice to aging-dependent progressive globerulopathy (Rosenberg et al, 2002) and increases Aβ(1-42) aggregation and deposition in mouse models of Alzheimer's disease (DeMattos et al, 2004). Furthermore, genome-wide association studies implicate clusterin in the development of Alzheimer's disease (Harold et al, 2009; Lambert et al, 2009; Wijsman et al, 2011). Small populations (<5%) of some ER chaperones, such as the HSP70 BiP and the lectin calreticulin, can be trafficked to the plasma membrane, particularly under stress or during apoptosis (Martins et al, 2010; Zhang et al, 2010). Protein disulfide isomerases (PDIs) can also be secreted to promote extracellular disulfide exchange (Hahm et al, 2013; Jordan & Gibbins, 2006). A role for these chaperones in extracellular proteostasis maintenance has not been demonstrated to date, rather their surface expression has been implicated in immunological signaling (Lee, 2014; Peters & Raghavan, 2011)."*
>
> Page 4, Paragraph 4: *"In contrast, the functional impact of UPR signaling on extracellular proteostasis capacity remains poorly defined. Not only are the established secreted chaperones not transcriptional targets of the UPR, but clusterin secretion is attenuated during conditions of ER stress, indicating that clusterin secretion is not a protective mechanism to regulate extracellular proteostasis in response to pathologic ER insults (Nizard et al, 2007). Similarly, ER stress reduces secretion of ER proteostasis factors such as protein disulfide isomerase (PDI), suggesting reduced extracellular regulation of disulfide integrity during ER stress (Terada et al, 1995). Thus, we sought to study the functional role for UPR signaling in adapting extracellular proteostasis capacity during conditions of ER stress."*
>
> Page 7, Paragraph 1, Line 8: *These results are consistent with previous results showing that negligible BiP is secreted to the extracellular space (Kern et al, 2009; Munro & Pelham, 1987; Yamamoto et al, 2003). Rather, BIP that evades the KDEL receptor is typically still retained at the cellular membrane (Wang et al, 2009; Zhang et al, 2010), as is common for other canonical ER-localized chaperones, particularly under apoptotic conditions (Jordan & Gibbins, 2006; Lee, 2014; Martins et al, 2010). HYOU1, the ER resident Hsp110 that both serves as a nucleotide exchange factor for BiP and displays its own chaperone function (Andreasson et al, 2010; Behnke & Hendershot, 2014), has not been implicated in either secretion or presentation at the cellular membrane."*
>
> Page 7, Paragraph 2, Line 9: "*These results also distinguish ERdj3 from other ER chaperones such as BiP or calreticulin, for which only a minor fraction of newly-synthesized protein is released into the extracellular space , even during stress (Dorner et al, 1987; Lodish & Kong,*

1990; Peters & Raghavan, 2011). Rather, the efficiency and selectivity of ERdj3 secretion indicates that its secretion is regulated by the canonical secretory pathway."

EDITOR COMMENT #2. "*Please also add further data to address the interplay between ERdj3 and BiP in secretion and association with misfolded client.*"

OUR RESPONSE TO EDITORIAL COMMENT #2. In the revised manuscript, we include new data showing that both ERdj3 and BiP co-purify with destabilized $^{FT}TTR^{A25T}$ in cellular lysates (see revised **Figure S6C**). Alternatively, only ERdj3 co-purifies with $^{FT}TTR^{A25T}$ in conditioned media. These results demonstrate that ERdj3 and BiP both function to dictate intracellular proteostasis for destabilized $^{FT}TTR^{A25T}$, but only ERdj3 has an important role in regulating extracellular $^{FT}TTR^{A25T}$ proteostasis. These results further support the data shown in **Figure 6**, demonstrating that the co-secretion of ERdj3-substrate interactions is dictated by the functional interplay between ERdj3 and BiP intracellularly, while highlighting the specific BiP-independent role for ERdj3 in regulating extracellular proteostasis. Furthermore, these results are consistent with new immunoblotting experiments showing that we do not detect BiP in conditioned media performed using an anti-BiP antibody (see revised **Figure S2C**). The changed text in the revised manuscript is found directly below and is discussed in additional detail in **Our Response to Reviewer #1 Comment #3**:

Page 9, Paragraph 1, Line 5: *"Longer conditioning under ATF6 activation (48 h) also failed to lead to immunodetectable BiP secretion (**Figure S2C**)."*

Page 15, Paragraph 1, Line 4: *" Despite a robust interaction between BiP and intracellular $^{FT}TTR^{A25T}$, BiP does not appear in extracellular ^{FT}TTR immunopurifications, consistent with our inability to observe extracellular BiP and supporting our model whereby extracellular ERdj3 functions independent of BiP (**Figure S6C**)."*

EDITOR COMMENT #3. "*Along the same lines, we would also ask you to follow the recommendations by ref#3 to look more closely at the disaggregation activity of secreted ERdj3 and whether this activity would occur during the physiological stress response.*"

OUR RESPONSE TO EDITORIAL COMMENT #3. In the revised manuscript, we show that conditioned media containing high levels of secreted ERdj3 decreases proteotoxicity of a toxic prion protein conformation (TPrP)(see revised **Figure 4**). Furthermore, we show that even the low levels of ERdj3 secreted from cells in the absence of protein overexpression are protective against TPrP induced proteotoxicity (see revised **Figure S4**). **Reviewer #3 Comment #1** asks us to complement this TPrP data with in vitro evidence that ERdj3 attenuates in vitro TPrP conversion. Unfortunately, there is not a comparable assay to measure the conversion of TPrP, as for Aβ fibrillization. TPrP is not an aggregated state of PrP, but rather a misfolded monomeric species possessing neither a known nor predicted spectroscopic signature. Thus, we were unable to use an aggregation inhibition assay for TPrP like the one we used to show that ERdj3 attenuates Aβ aggregation (see revised **Figure 3B-D**).

We also tried the experiment explicitly brought up in **Reviewer #3 Comment #1** to demonstrate that cell-secreted ERdj3 protects cells from Aβ proteotoxicity. Unfortunately, despite Aβ(1-42) inducing a modest ~15% reduction in SK-N-SH cell viability, conditioned media prepared on control cells not overexpressing ERdj3 significantly attenuated the viability of these cells and completely abolished Aβ-induced proteotoxicity in these cells. Thus, we were unable to perform the experiment suggested by the reviewer. Our use of TPrP in the original submission was ideal because it is one of the few toxic aggregation-prone proteins that can induce cell proteotoxicity at low nM concentrations (e.g., Aβ toxicity requires >50 μg/mL Aβ) and was able to induce proteotoxicity in the presence of conditioned media, allowing us to explicitly demonstrate the capacity for physiologically-relevant levels of secreted ERdj3 to attenuate proteotoxicity of a misfolded, toxic protein conformation, as suggested by the editor (see revised **Figure 4 & S4**).

To further address the reviewer's comment, we have been adapting a recently-reported *C. elegans* proteotoxicity assay (Diomede et al Blood 2014) to evaluate the capacity for ERdj3 to inhibit the toxicity of cell-secreted aggregation-prone proteins such as TTR^{A25T}. In this experiment, we are using conditioned media prepared on cells overexpressing destabilized TTR^{A25T} and/or ERdj3 to induce proteotoxicity in *C. elegans* that

can be measured by pharyngeal pumping. Our focus on developing this assay, which derives from the comments of Reviewer #3, allows us to explicitly evaluate the capacity for UPR-dependent increases in ERdj3 to attenuate proteotoxicity in a physiologically-relevant, experimentally-tractable model system. This system will allow us to measure toxicity induced by modest levels of secreted proteins, significantly less than the extremely high concentrations of recombinant proteins (e.g., Aβ) commonly used for such assays. This system will be ideal to address the involvement of physiologically-relevant levels of ERdj3 secretion induced by UPR activation on the proteotoxicity of cell-secreted proteins. Initial experiments show that ERdj3 secretion attenuates the proteotoxicity of cell-secreted TTR^{A25T}, suggesting that ERdj3 is protective against TTR^{A25T} toxicity in the extracellular space. Since this assay, which has not been previously applied to complex media nor to TTR, requires extensive additional experimentation to demonstrate the proteotoxicity of cell-secreted TTR^{A25T} and the capacity for ERdj3 to attenuate proteotoxic TTR aggregation, we feel that it will be better to report these results in a follow-up manuscript where we can discuss at length the establishment of this assay and all of the appropriate controls necessary to confirm the results observed in our initial experiments. We are highly motivated to continue the development and implementation of this assay to address the very exciting points brought up by **Reviewer #3**.

In the current manuscript, we address the spirit of **Reviewer #3 Comment #1** and **Editor Comment #3** by providing further discussion of the link between UPR signaling and extracellular proteostasis regulation in human protein misfolding diseases (see **Our Response to Reviewer #1 Comment #1**). Additionally, we have tempered our language throughout the revised manuscript regarding our own work to be more cautious when discussing the potential protective benefits of cell-secreted ERdj3 in attenuating proteotoxicity of extracellular proteins. An example of this type of change is included below:

> Page 5, Paragraph 2, Line 8: "*Furthermore, we demonstrate that ERdj3 can co-secrete with destabilized, misfolding-prone clients under conditions where the ER HSP70 chaperoning pathway is overwhelmed, preemptively chaperoning these misfolding-prone secreted proteins that evade ER quality control in the extracellular environment. Thus, the capacity for ERdj3 to function in both ER and extracellular proteostasis provides an unanticipated direct link between these two environments that is regulated by the UPR during conditions of ER stress.*"

Furthermore, we would like to highlight that we have provided significant additional data related to the mechanism by which secreted ERdj3 increases extracellular proteostasis capacity. We now show that the ERdj3 mutant H53Q binds to denatured RNAse A in the extracellular space, indicating that ERdj3 binding to misfolded proteins in the extracellular environment is independent of its role as a co-chaperone for BiP (see revised **Figure 3A**). Additionally, we show that secreted, destabilized proteins such as TTR^{A25T} efficiently bind to both BiP and ERdj3 intracellularly, but only ERdj3 in the extracellular space, indicating that ERdj3 is an important extracellular chaperone involved in binding to destabilized TTR (see revised **Figure S6C**). The inability for TTR^{A25T} to co-immunopurify BiP in the extracellular space is consistent with the lack of secreted BiP in the extracellular space, which we further demonstrate in our revised manuscript (**Figure S2C**). These new data help to address the mechanism by which secreted ERdj3 increases the extracellular environment during conditions of ER stress. Further discussion of these new data are included below in **Our Response to Reviewer #2 Comment #2**.

REVIEWER #1

Reviewer #1 General Comments: *"This paper is well-written, it represents a very clear story and the overall structure of the manuscript is well organized. The phenotypes that authors show are robust and convincing. Also, the conclusions of all experiment are fair in their interpretations. This manuscript could be accepted after minor revisions."*

Our Response to Reviewer #1 General Comment. We thank the reviewer for taking the time to review our manuscript and provide constructive feedback, which we address below.

Reviewer #1 Comment #1: *"In order to improve the impact of this manuscript, I think it may be interesting to perform some experiment supporting the principal concept, and highlight the biomedical application. If possible, a correlation between ERdj3 expression in and amyloid beta deposition could be provided using brain tissue derived from AD or JCD patients."*

Our Response to Reviewer #1 Comment #1: Previous publications have shown that the UPR is activated in tissues isolated from Alzheimer's disease and Creutzfeld-Jacob disease patients. Since ERdj3 is upregulated as part of the UPR transcriptional program, *ERdj3* expression is increased during these diseases. These results suggest that UPR-dependent increases in ERdj3 could provide a protective mechanism to protect cells from extracellular protein aggregation. Furthermore, previous work has shown that mutations in presenilin that predispose individuals to Alzheimer's disease significantly impair activation of the ATF6 arm of the UPR – the arm we show to be responsible for regulating extracellular ERdj3 (see revised **Figure 2A-E**). This suggests that the decreased capacity to regulate extracellular ERdj3 through ATF6 predisposes individuals to extracellular protein aggregation associated with this disease. We explicitly discuss this in the revised manuscript, as described below:

> Page 17, Paragraph 2, Line 1: *"The capacity of UPR-dependent activation of ATF6 to influence extracellular proteostasis through ERdj3 secretion indicates a potential role for this pathway in extracellular protein aggregation pathologies. UPR signaling is activated in many extracellular protein aggregation diseases, including Alzheimer's disease and the systemic amyloidoses (Hetz & Mollereau, 2014; Hoozemans et al, 2012; Saxena et al, 2009; Teixeira et al, 2006). Our results showing that secreted ERdj3 can increase the extracellular chaperoning and prevent extracellular aggregation and/or proteotoxicity of disease-associated proteins such as TPrP, TTR^{A25T}, and Ab suggest that ATF6-dependent regulation of ERdj3 secretion is a potential mechanism to protect the extracellular space from proteotoxicity. Consistent with this prediction, presenilin mutations causatively associated with Alzheimer's disease significantly impair ATF6 activation during stress (Katayama et al, 2001; Katayama et al, 1999). Thus, a decreased capacity to regulate extracellular proteostasis through ATF6 activation and consequent ERdj3 secretion could be a contributing factor in the disease pathology of patients harboring these mutations."*

As suggested by the reviewer, we are now correlating ERdj3 expression and intra- and extracellular protein levels with various markers of protein aggregation in patient samples and in animal models of these diseases. While we are highly motivated to continue these studies, which will take some time in order to get enough samples to render the results statistically significant, we feel that these types of correlation, while important, are outside the scope of this initial report.

Reviewer #1 Comment #2: *"The authors should discuss other papers showing secretion of ER foldases. The way in which the study is presented gives the impression that this is the first chaperone described to be secreted, but this is not the case. PDI and ERp57 have been shown to be decreased and even participate in the extracellular folding of proteins and there are even reviews on the subject (see Antioxid Redox Signal. 2006 Mar-Apr;8(3-4):312-24.). BiP has been also shown to express at the extracellular membrane, and has been used for cancer immunotherapy. Finally, many papers from Guido Kroemer"*

Our Response to Reviewer #1 Comment #2: As suggested by the reviewer, we now include discussion of other UPR target proteins shown to be trafficked from the ER during conditions of ER stress. This includes significant discussion contrasting the underlying mechanisms of ERdj3 secretion (described in our manuscript) with the secretion of these other ER proteostasis factors. These changes are included in the manuscript, as below.

> Page 3, Paragraph 2, Line 8: *"Small populations (<5%) of some ER chaperones, such as the HSP70 BiP and the lectin calreticulin, can be trafficked to the plasma membrane, particularly under stress or during apoptosis (Martins et al, 2010; Zhang et al, 2010). Protein disulfide isomerases (PDIs) can also be secreted to promote extracellular disulfide exchange (Hahm et al, 2013; Jordan & Gibbins, 2006). A role for these chaperones in extracellular proteostasis maintenance has not been demonstrated to date, rather their surface expression has been implicated in immunological signaling (Lee, 2014; Peters & Raghavan, 2011)."*

> Page 4, Paragraph 4, Line 1: *"In contrast, the functional impact of UPR signaling on extracellular proteostasis capacity remains poorly defined. Not only are the established secreted chaperones not transcriptional targets of the UPR, but clusterin secretion is attenuated during conditions of ER stress, indicating that clusterin secretion is not a protective mechanism to regulate extracellular proteostasis in response to pathologic ER insults (Nizard et al, 2007). Similarly, ER stress reduces secretion of ER proteostasis factors such as protein disulfide isomerase (PDI), suggesting reduced extracellular regulation of disulfide integrity during ER stress (Terada et al, 1995). Thus, we sought to study the functional role for UPR signaling in adapting extracellular proteostasis capacity during conditions of ER stress."*

> Page 7, Paragraph 1, Line 4: *"In stark contrast to ERdj3, BiP and HYOU1, two abundantly expressed, UPR-induced, ER chaperones, were not detected in the conditioned media (**Figure 1C**), reflecting the presence of ER retention motifs on these proteins. These results are consistent with previous results showing that negligible BiP is secreted to the extracellular space (Kern et al, 2009; Munro & Pelham, 1987; Yamamoto et al, 2003). Rather, BIP that evades the KDEL receptor is typically still retained at the cellular membrane (Wang et al, 2009; Zhang et al, 2010), as is common for other canonical ER-localized chaperones, particularly under apoptotic conditions (Jordan & Gibbins, 2006; Lee, 2014; Martins et al, 2010). HYOU1, the ER resident Hsp110 that both serves as a nucleotide exchange factor for BiP and displays its own chaperone function (Andreasson et al, 2010; Behnke & Hendershot, 2014), has not been implicated in either secretion or presentation at the cellular membrane. Alternatively, intracellular levels of BiP and HYOU1 were significantly increased upon Tg treatment. These data indicate that increased extracellular ERdj3 levels result from constitutive secretion and not from leakage of ER proteins into the extracellular space, as has been proposed for other ER chaperones (Booth & Koch, 1989)."*

> Page 7 Paragraph 2, Line 9: *"These results further demonstrate that ERdj3 secretion is not a consequence of cell death or "leaky" release of ER proteins into the cellular media. These results also distinguish ERdj3 from other ER chaperones such as BiP or calreticulin, for which only a negligible fraction of newly-synthesized protein is released into the extracellular space, even during stress (Dorner et al, 1987; Lodish & Kong, 1990; Peters & Raghavan, 2011). Rather, the efficiency and selectivity of ERdj3 secretion indicates that its secretion is regulated by the canonical secretory pathway."*

Additionally as suggested by the reviewer, we have adapted the abstract to better reflect the novelty of UPR-dependent increases in ERdj3 secretion relative to these other proteins. Specifically, we highlight that ERdj3 remains the first report of a chaperone whose secretion into the extracellular space is induced by the UPR and whose co-secretion in complex with misfolding-prone proteins is regulated by the capacity for ER quality control. This change is included in the revised manuscript, as below:

Page 2, Paragraph 1, Line 11: *"This regulated co-secretion of ERdj3 with misfolded clients directly links ER and extracellular proteostasis during conditions of ER stress. ERdj3 is, to our knowledge, the first metazoan chaperone whose secretion into the extracellular space is regulated by the UPR, revealing a new mechanism by which UPR activation regulates extracellular proteostasis."*

Reviewer #1 Comment #3: *"A non-functional ERdj3 (instead ERdj3-KDEL) could be used in experiment related with aggregation of Abeta (Figure 3) or the PrP toxicity (Figure 4). ERp57 has been shown to have functions independent of the enzymatic activity."*

Our Response to Reviewer #1 Comment #3: We believe the reviewer is addressing the potential for ERdj3 to influence extracellular proteostasis through mechanisms independent of its functional, intracellular role as an HSP40. We have performed additional experiments to demonstrate that ERdj3 functions independent of its role as an HSP40. In the revised manuscript, we now show that secreted ERdj3^{H53Q}, which disrupts the functional interaction between BiP and ERdj3, selectively binds denatured RNAse, directly demonstrating that the binding of secreted ERdj3 to denatured proteins is independent of its HSP40 activity (**Figure S4A**). This new result is discussed in the revised manuscript, as below:

Page 10, Paragraph 2, Line 8: "*The capacity for ERdj3^{H53Q} to similarly associate with denatured RNAse resin indicates that secreted ERdj3 interacts with denatured substrates through a mechanism largely independent of its intracellular role as an HSP40 co-chaperone that delivers misfolded substrates to BiP for ATP-dependent chaperoning."*

Additionally, we immunopurified the destabilized ^{FT}TTR^{A25T} from both cell lysates and media and monitored the co-purification of ERdj3 and BiP. Intracellularly, we observe a strong association between ^{FT}TTR^{A25T} and both ERdj3 and BiP, reflecting the functional interplay between ERdj3 and BiP in the intracellular folding and trafficking of destabilized TTRs, as previously reported (**Figure S6C**). In contrast, only ERdj3 co-purifies with ^{FT}TTR^{A25T} in media (see revised **Figure S6C**). This is consistent with the lack of significant BiP populations in conditioned media, as shown in **Figures 1C & S2C** of the revised manuscript. We discuss these new immunopurification results in the revised manuscript, as below:

Page 15, Paragraph 1, Line 4: "*Despite a robust interaction between BiP and intracellular ^{FT}TTR^{A25T}, BiP does not appear in extracellular ^{FT}TTR immunopurifications, consistent with our inability to observe extracellular BiP and supporting our model whereby extracellular ERdj3 functions independent of BiP (**Figure S6C**)."*

Finally, the reviewer asked for us to perform TPrP toxicity assays using media prepared on cells overexpressing ERdj3^{KDEL}. In cells overexpressing ERdj3^{KDEL}, extracellular ERdj3 levels are typically ~5-fold higher than control cells (albeit lower than those observed for ERdj3 overexpressing cells), significantly challenging the experiment proposed by the reviewer. This results from the displacement of endogenous intracellular ERdj3 with ERdj3^{KDEL}. To address the reviewer's concern, we would like to highlight that we explicitly show that TPrP toxicity is significantly attenuated when cells are incubated in media conditioned on cells overexpressing ERdj3 (see **Figure 4B,C** of the revised manuscript). Additionally, we show that TPrP toxicity is exacerbated on cells cultured in conditioned media prepared on cells RNAi-depleted of ERdj3 (see revised **Figure S4B,C**). Thus, we are able to show that high levels of ERdj3 and ablation of ERdj3 have opposing effects on TPrP toxicity, strongly supporting the functional role for ERdj3 in protecting cells from proteotoxic protein conformations.

REVIEWER #2.

Reviewer #2 Comment #1: *"Collectively, this an impressive body of work but direct proof of whether a specific substrate ferried by ERdj3 is prevented from aggregating when bound to ERdj3 extracellularly is not shown. While an aberrant polypeptide, e.g., TTR A25T, is clearly a substrate, does the chaperone prevent the toxic effects of this molecule once secreted? The other experiments, noted above, in which ERDj3 prevented aggregation and/or toxicity used other substrates. Therefore, the authors should be cautious about their conclusions that ERdj3 association in the cell and then secretion "protects" the extracellular compartment."*

Our Response to Reviewer #2 Comment #1. We have moderated our description of co-secretion being protective in the abstract and the discussion, to make it clear that while we reasonably expect this to be a protective mechanism, based on the general protective nature of holdase chaperones, that nevertheless it is still speculative at this point. This is discussed in the revised manuscript, as described below:

> Page 2, Paragraph 1, Line 8 : "*Moreover, ERdj3 can co-secrete with destabilized, aggregation-prone proteins in a stable complex under conditions where ER chaperoning capacity is overwhelmed, preemptively providing extracellular chaperoning of proteotoxic misfolded proteins that evade ER quality control.*"
>
> Page 17, Paragraph 1, Line 2: "*Simultaneously, increased intracellular ERdj3 is available to bind and co-secrete with misfolding-prone protein clients that evade ER quality control and traverse the secretory pathway, providing preemptive chaperone capacity to the extracellular space. Importantly, clusterin co-secretion, unlike ERdj3 co-secretion, assists destabilized clients in evading ER quality control, providing a potential reason for the reduced secretion of clusterin during conditions of ER stress.*"

Reviewer #2 Comment #2. *"A second point concerns the question of whether ERdj3 is acting alone or in a complex with other chaperones. The effect of conditioned media, as used for the TTR studies, fails to differentiate between these scenarios. However, BiP is not secreted upon ATF6 activation (Fig. 2A), although a BiP antibody (instead of the anti-KDEL antibody) should be used to confirmed this observation."*

Our Response to Reviewer #2 Comment #2: We have added an additional panel (**Figure S2C**) wherein we demonstrate that we continue to not observe extracellular BiP in response to ATF6 or XBP1s activation, even after a much longer (48 h) induction period. As suggested by the reviewer, we used an anti-BiP antibody for these experiments. This new experiment is discussed in the revised manuscript, as below:

> Page 9, Paragraph 1, Line 1: "*Longer conditioning under ATF6 activation (48 h) also failed to lead to immunodetectable BiP in conditioned media (**Figure S2C**).*"

Furthermore, we performed an immunopurification of $^{FT}TTR^{A25T}$ secreted from $HEK293^{DAX}$ cells co-overexpressing ERdj3 (**Figure S6C**). In these experiments, we demonstrate that ERdj3, but not BiP, co-purifies with $^{FT}TTR^{A25T}$ under these conditions. This is in contrast to IPs from $^{FT}TTR^{A25T}$ from cell lysates where we (and others) observe a strong interaction between BiP and $^{FT}TTR^{A25T}$. This result indicates that ERdj3 does not deliver destabilized $^{FT}TTR^{A25T}$ to BiP in the extracellular environment, indicating that ERdj3 functions extracellularly independent of its role as a BiP co-chaperone. These new results are included in the revised manuscript text found below:

> Page 15, Paragraph 1, Line 4: *"Despite a robust interaction between BiP and intracellular $^{FT}TTR^{A25T}$, BiP does not appear in extracellular ^{FT}TTR immunopurifications, consistent with our inability to observe extracellular BiP and supporting our model whereby extracellular ERdj3 functions independent of BiP (**Figure S6C**)."*

Reviewer #2 Comment #3: *"Interestingly, BiP is secreted from yeast exposed to UPR inducing conditions (Barlowe and colleagues, Mol. Biol. Cell, 2001; Miller and colleagues, Genetics 2009); therefore, the last*

sentence of the abstract needs to be slightly modified and this fact should be mentioned. Is there any evidence of extracellular BiP when the TTR-ERdj3 complex was observed extracellularly?"

Our Response to Reviewer #2 Comment #3. As suggested by the reviewer, we have adapted the last sentence of the abstract to highlight the unique functions of ERdj3 co-secretion highlighted by our work. Specifically, we highlight that ERdj3 remains the first report of a metazoan chaperone whose secretion into the extracellular space is induced by the UPR and whose co-secretion in complex with misfolding-prone proteins is regulated by the capacity for ER quality control. This change is included in the revised manuscript, as below:

> Page 2, Paragraph 1, Line 12: *"ERdj3 is, to our knowledge, the first metazoan chaperone whose secretion into the extracellular space is regulated by the UPR, revealing a new mechanism by which UPR activation regulates extracellular proteostasis."*

Furthermore, as discussed in **Our Response to Reviewer #2 Comment #2**, we have performed additional immunoblotting and an immunopurification of ^{FT}TTR^{A25T} secreted from HEK293^{DAX} cells co-overexpressing ERdj3 and demonstrate that we find no extracellular BiP or BiP co-purifying with ERdj3 under these conditions. These results strongly indicate that ERdj3 does not function as a conventional HSP40 in the extracellular space. This change in the revised manuscript is discussed above in **Our Response to Reviewer #2 Comment #2.**

Reviewer #2 Comment #4: *"In addition, data suggesting that BiP affects ERdj3-TTR interaction is indirect: Fig. 6 seems to lack data indicating that the levels of BiP rise. More definitive results might be obtained if there is an effect when BiP is silenced, perhaps by shRNA or subtilase toxin administration. Addressing some of these minor points would significantly strengthen the authors' conclusions. "*

Our Response to Reviewer #2 Comment #4: We thank the reviewer for this suggestion. In our revised **Figure 6A**, we now show that stress-independent ATF6 activation significantly increases intracellular BiP levels. Additionally, we show in **Figure 6C** of the revised manuscript increases in intracellular BiP observed upon BiP overexpression. These results directly address the comment of the reviewer to demonstrate increases in intracellular BiP levels induced by these treatments.

It has been shown that reduction in *BiP* by RNAi significantly activates the UPR (see Li et al, Cell Death Diff., 2008, doi: 10.1038/cdd.2008.81). Similarly, BiP inhibition afforded by subtilisin is also known to activate the UPR (Paton et al, Nature, 2006, doi: 10.1038/nature05124) The increase in UPR signaling afforded by *BiP* shRNA or subtilisin significantly convolutes using these approaches to define the specific role for BiP in defining ERdj3-substrate co-secretion.

Nonetheless, to address the spirit of the reviewers suggestion, we used protein overexpression and a ERdj3 mutant deficient in BiP binding to demonstrate the critical role for functional ERdj3-BiP interactions in defining co-secretion of ERdj3-substrate complexes. In our revised **Figure 6A,B**, we show that the secretion of ERdj3^{WT}-^{FT}TTR^{A25T} complexes are reduced upon stress-independent activation of ATF6 – the UPR-associated transcription factor responsible for increasing *BiP* expression. Additionally, we show that overexpression of *BiP* significantly reduces secretion of ERdj3^{WT}-^{FT}TTR^{A25T} complexes (**Figure 6C,D**). These effects were completely abrogated by overexpression of ERdj3^{H53Q} mutants that disrupt the functional interaction between BiP and ERdj3. Similar results were observed for the secretion of ERdj3-APP complexes (**Figure 6E**). We feel that these results directly demonstrate the functional importance for intracellular ERdj3-BiP interactions in defining the co-secretion of ERdj3-substrate co-secretion.

Reviewer #2 Comment #5: *"Regardless of these concerns, this manuscript reports on a new and important phenomenon in an active (and medically relevant) area of research. The quality of the data is excellent and the myriad tools and systems used by the investigators is a notable strength of the manuscript. The paper will be an excellent addition to the journal. "*

Our Response to Reviewer #2 Comment #5: We appreciate the time the reviewer put into reviewing our manuscript and providing critical feedback for our work, which we address above.

Reviewer #2 Minor Comments. The reviewer also included 9 minor comments, which we address below.

Reviewer #2 Minor Comment #1: "*p. 5, last full paragraph, it is confusing that HEK293T cells are again listed amongst the other cell lines (since the cells were already discussed and used). This is especially relevant since the signal is so much weaker in Fig. S1C compared to Fig. 1A."*

Our Response to Reviewer #2 Minor Comment #1: As suggested by the reviewer, we removed HEK293T cells from the list of other cells included on Page 5 of our original manuscript. The revised manuscript has been adapted as indicated below:

> Page 6, Paragraph 3, Line 7: *"Endogenous ERdj3 was also detected in conditioned media collected from a variety of human cell lines, including HepG2, Huh7, SH-SY5Y, and HeLa (**Figure S1C**)."*

Reviewer #2 Minor Comment #2: "*The time point used to obtain the data in Fig. S2A is not given. Also, is BiP secreted if longer ATF6 (or XBP1) induction times are used (viz. Fig, 2A)?"*

Our Response to Reviewer #2 Minor Comment #2: We have added the time point (6 h) to the **Fig. S2A** caption. We do not see BiP secretion at longer ATF6/XBP1s inductions (see new **Fig S2C**). This alteration is included in the revised manuscript, as below:

> Page 9, Paragraph 1, Line 5: "*Longer conditioning under ATF6 activation (48 h) also failed to lead to immunodetectable BiP in conditioned media (**Figure S2C**)."*

Reviewer #2 Minor Comment #3: "*As reported in Zhou et al., 2012, the vacuolization study could be buttressed by the use of another reporter for TPrP (toxic) effects in the neuroblastoma cell line."*

Our Response to Reviewer #2 Minor Comment #3: The vacuolarization phenotype is particularly sensitive, enabling a clear result in these initial studies. We anticipate more extensive follow-up experiments in which we will explore a wider range of phenotypes.

Reviewer #2 Minor Comment #4: "*In Fig. 5B, why is the level of TTR A25T in the IB so weak when probing with anti-FLAG/M2? This is not the case in other experiments when co-expression is being analyzed (compare for example Fig. 5B and C)."*

Our Response to Reviewer #2 Minor Comment #4: Here as well, the apparent weak signal from A25T is do to low imaging gain to accommodate the much more intense relative signal from the more efficiently secretion WT-TTR. The more efficient secretion of TTR^{WT} relative to TTR^{A25T} has been reported previously by our lab (Sekijima et al, Cell, 2006 and Shoulders et al, Cell Reports, 2013).

Reviewer #2 Minor Comment #5: "*p. 12, "significantly" should be deleted since the effect of Tg pretreatment changes the relative recovery of ERdj3 only modestly (but yes, the data are statistically significant...)"*

Our Response to Reviewer #2 Minor Comment #5: We have removed the word "significantly", noting that it is indeed a modest, though reproducible, effect. This alteration is included in the revised manuscript as below:

> Page 15, Paragraph 2, Line 3: *"Despite reducing ${}^{FT}TTR^{A25T}$ secretion through ER stress-dependent UPR activation (Shoulders et al, 2013), pretreatment with Tg increased the relative recovery of secreted ERdj3 in ${}^{FT}TTR^{A25T}$ immunopurifications (**Figure S6A,B**). This ER stress-induced change in the association between ERdj3 and its clients suggests that ER proteostasis network components are involved in regulating ERdj3 co-secretion."*

Reviewer #2 Minor Comment #6: "*Fig. 2C and E. Although p values are given in the legend, I failed to note asterisks in the figures, which are supposed to reflect the provided statistics."*

Our Response to Reviewer #2 Minor Comment #6: We apologize for omitting these asterisks in the initial submission. We now include the appropriate asterisks in the revised **Figure 2C-E**.

Reviewer #2 Minor Comment #7: "*HYOU1 is not a commonly known chaperone so additional information on its function should be provided."*

Our Response to Reviewer #2 Minor Comment #7: We thank the reviewer for pointing out the lack of information regarding HYOU1. In our revised manuscript, we now include additional discussion of HYOU1, specifically highlighting its functional role as a nucleotide exchange factor in the BiP chaperoning pathway. This revision is found in the new manuscript, as below:

> Page 7, Paragraph 1, Line 13: *"HYOU1, the ER resident Hsp110 that both serves as a nucleotide exchange factor for BiP and displays its own chaperone function (Andreasson et al, 2010; Behnke & Hendershot, 2014), has not been implicated in either secretion or presentation at the cellular membrane."*

Reviewer #2 Minor Comment #8: "*Fig. 3D. The administration of BSA yields an effect that is intermediate between the buffer control and ERdj3. Quantitative data need to be provided for replicates of this experiment, or another control should be used."*

Our Response to Reviewer #2 Minor Comment #8: We use BSA as a control protein, as it is protein known to exhibit non-specific interactions with proteins and small molecules and is commonly used as a control for protein aggregation experiments such as those included here. The ability for BSA to modestly inhibit Abeta aggregation at high concentrations is consistent with previous reports, which we now cite in the revised manuscript. We discuss these previous results in the revised manuscript as described below:

> Page 10, Paragraph 3, Line 9: *"The control serum protein bovine serum albumin (BSA), a protein known to modestly inhibit Aβ aggregation (Bohrmann et al, 1999; Milojevic et al, 2007; Milojevic et al, 2009; Reyes Barcelo et al, 2009), did not dramatically influence $A\beta_{1-40}$ aggregation at a equivalent molality (**Figure 3C**)."*

Our use of BSA also demonstrates the significant capacity for ERdj3 to inhibit Abeta aggregation substoichiometry, even when compared to a protein known to exhibit broad non-specific binding. Furthermore, the curves shown in **Figure 3D** are averages of 3 independent experimental points, which provide highly reproducible results across multiple experiments.

Reviewer #2 Minor Comment #9: "*Fig. S2. The arrow for Grp94 does not indicate a specific protein species."*

Our Response to Reviewer #2 Minor Comment #9: We apologize for the arrows shown in **Figure S2E** of the original submission. We have corrected this issue in the revised **Figure S2E**.

REVIEWER #3.

Reviewer #3 General Comments.

"Overall, I find this a highly interesting data set and the paper is well-written. In my view, the data are fully conclusive to show ERdj3 co-secretion with unfolded clients and suggest that this indeed may be a backup mechanism under conditions of BiP shortage (and thus impaired ERAD) in order to dump misfolded clients into the extracellular milieu. Regarding the suggested other role of ERdj3 in regulating extracellular proteostasis, I have some questions that remain to be solved."

Our Response to Reviewer #3 General Comment. We thank the reviewer for taking the time to review our manuscript and provide critical comments on our work. We address all of the reviewer's valuable comments below. We would also like to address the reviewer to **Our Response to Editor Comment #3**, where we provide additional information regarding alterations to the text related to **Reviewer #3 Comment #1 & 2**.

Reviewer #3 Comment #1. "*1. The finding that recombinant ERdj3 has strong anti-aggregation power against e.g. Abeta (1-40) is exciting in itself and of course leads to the suggestion that secreted ERdj3 may have protective effects in Alzheimer's disease. It would first of all have been nice(r) to show that also medium from ERdj3 over expressing cells would protect against toxicity from Abeta peptides rather than from toxicity of PrP. Alternatively, the cellular data with PrP could have been complemented with effects of ERdj3 on in vitro PrP aggregation (rather than Abeta)."*

Our Response to Reviewer #3 Comment #1. There is not a comparable assay to measure the conversion of TPrP conformation as there is for Aβ fibrillation. As suggested by the reviewer, we attempted to perform the experiment suggested by the reviewer to demonstrate that '*medium from ERdj3 overexpressing cells would protect against Abeta peptides…"*. Unfortunately, despite Aβ(1-42) inducing a modest ~15% reduction in SK-N-SH cell viability, conditioned media prepared on control cells not overexpressing ERdj3 signficantly attenuated the viability of these cells and completely abrogated Aβ-induced toxicity. Thus, we were unable to perform the experiment suggested by the reviewer. Our use of TPrP in the original submission was ideal because it is one of the few toxic aggregation-prone proteins that can induce cell proteotoxicity at low nM concentrations (e.g., Aβ toxicity requires >50 µg/mL Abeta) and was able to induce measurable proteotoxicity in the presence of conditioned media (see revised **Figure 4 & S4**).

Alternatively, we have been adapting a recently reported *C. elegans* proteotoxicity assay (Diomede et al, Blood 2014) to evaluate the capacity for ERdj3 to inhibit the toxicity of secreted amyloidogenic proteins such as TTR^{A25T}. Initial experiments indicate that ERdj3 attenuates TTR^{A25T} proteotoxicity, although these studies are ongoing. Since this assay requires significant additional experimentation to demonstrate the proteotoxicity of cell-secreted TTR^{A25T} and the capacity for ERdj3 to attenuate aggregation, we feel that it will be better to report these results in a follow-up manuscript where we can discuss at length the establishment of this assay and all of the appropriate controls necessary to confirm the results observed in our initial experiments. We further address the importance of developing this assay and additional changes to our manuscript related to this comment above in **Our Response to Editor Comment #3.**

Reviewer #3 Comment #2.*"2. The cellular data on PrP aggregation being attenuated by media from ERdj3 over expressing cells requires a number of additional controls. To proof that it is mediated by excreted (free) ERdj3, anti-body blocking experiments are required. In addition, recombinant ERdj3 could be used as positive control and media collected from ERdj3-KDEL over expressing cells as additional negative control. "*

Our Response to Reviewer #3 Comment #2. More detailed mechanistic studies on the protection of cells from TPrP toxicity by ERdj3 are the subject of future experiments. Antibody blocking experiments are not feasible because we have found that available ERdj3 antibodies are unable to immunoprecipitate native ERdj3 bound to substrate. As to performing TPrP toxicity assays using media prepared on cells overexpressing $ERdj3^{KDEL}$, these cells have extracellular ERdj3 levels typically ~5-fold higher than control cells (albeit significantly lower than those observed for ERdj3 overexpressing cells), challenging the experiment proposed

by the reviewer. This results from the displacement of endogenous intracellular ERdj3 with ERdj3^{KDEL} and is evident from our experiment shown in revised **Figure 1A**, where media levels of ERdj3 in ERdj3^{KDEL} overexpressing cells are higher than those for mock-transfected cells. To address the reviewer's concern, we would like to highlight that we explicitly show that TPrP toxicity is significantly attenuated when cells are incubated in media conditioned on cells overexpressing ERdj3 (see **Figure 4B,C** of the revised manuscript). Additionally, we show that TPrP toxicity is exacerbated on cells cultured in conditioned media prepared on cells RNAi-depleted of *ERdj3* (see revised **Figure S4B.C**). This effectively serves as the requested negative control for this experiment. Thus, we are able to show that high levels of ERdj3 and ablation of ERdj3 have opposing effects on TPrP toxicity, strongly supporting the functional role for ERdj3 in protecting cells from proteotoxic protein conformations.

Reviewer #3 Comment #3. "*3. I wonder what the evidence is that ERdj3 can leave the ER without clients such that it is available for extracellular chaperoning. On one hand, the authors state that client release may not happen extracellularly because BiP is not out there (I think this is a valid argumentation). On the other hand, conditions that induce ERdj3 excretion are usually such that they will involve co-excreting with clients, meaning no free chaperone will be present in the extracellular milieu. I wonder whether this actually explains why only co-expression and not co-incubation results in IP-interaction between mutant TTR and ERdj3 (figure 5? I realise that the data with the conditioned medium in figure 4 are suggesting otherwise, but one may argue that the preparations of the conditioned medium in these experiments artificially induces the release of clients from ERdj3. Is there any manner by which the authors can exclude this possibility?"*

Our Response to Reviewer #3 Comment #3. We show that free TTR^{A25T} and ERdj3 do interact in the extracellular space (see revised **Figure S5A**). This interaction is not RIPA-resistant, as is the case for the co-secreted complex, as the reviewer suggests. Also, in addition to the functional effects described in **Fig. 4**, we also demonstrate that secreted ERdj3 binds to denatured RNAse beads (**Fig. 3A**), demonstrating that the secreted protein is able to bind denatured clients. We are currently performing more detailed structural and mechanistic characterization of secreted ERdj3 for future manuscripts, but we cannot yet rule out the possibility that secreted ERdj3 does have some co-secretion partners that it does not bind as strongly as those studied in the present work.

Reviewer #3 Comment #4. *"4. Figure 3D: I am somewhat disturbed by the strong effect of BSA in this panel: can the authors explain this (minor comment)"*

Our Response to Reviewer #3 Comment #4. As discussed in **Our Response to Reviewer #2 Minor Comment #8**, BSA is well-established control to inhibit Abeta aggregation at high concentrations and is often used as a control for protein aggregation assays such as those described in **Figure 3B-D** and **Figure S3F** of the revised manuscript. We address this previous work in the revised manuscript, as described below:

> Page 10, Paragraph 3, Line 9: *"The control serum protein bovine serum albumin (BSA), a protein known to modestly inhibit Aβ aggregation (Bohrmann et al, 1999; Milojevic et al, 2007; Milojevic et al, 2009; Reyes Barcelo et al, 2009), did not dramatically influence $A\beta_{1-40}$ aggregation at a equivalent molality (**Figure 3C**)."*

Acceptance 23 September 2014

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by two of the original referees (comments included below) and as you will see they both find that all main criticisms have been adequately addressed. The only remaining point is that ref#3 asks you to clarify/rephrase the discussion of free ERdj3 being available for chaperoning misfolded proteins in the extracellular space. I am therefore happy to inform you that your manuscript has been accepted for publication with us, pending a slight text revision to address this minor concern.

Before we can proceed to transfer the manuscript for production I would also ask you to address the following editorial points:

-> We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format. The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data".

-> As of Jan 1st 2014 every paper published in The EMBO Journal includes a 'Synopsis' to further enhance its discoverability. The synopsis consists of a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to include your suggestions for bullet points.

You are welcome to send the bullet points, source data files and the revised manuscript text file (in word format) to me by email and we will then upload everything in house.

REFEREE REPORTS:

Referee #2:

The authors have done a terrific job at addressing the issues that were previously raised. This is an important and interesting study.

Referee #3:

The authors have adequately addressed most of my comments although I regret that some of the experiments I suggested apparently were not feasible.

Regarding my original comment #3, however, I still have a remaining comment. I am not arguing that the TTRA25T and ERdj3 interaction is RIPA-resistant. However, what I am trying to argue is that in vivo the ERdj3 will be released into the extracellular space bound to its clients. As no extracellular BiP will be present (neither RIPA, of course) how can ERdj3 be released from its clients under such "real" conditions to be available as chaperone for e.g. extracellular Abeta?