ERdj3 regulates BiP occupancy in living cells

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Summary
Co-chaperones regulate chaperone activities and are likely to impact a protein-folding environment as much as the chaperone itself. As co-chaperones are expressed substoichiometrically, the ability of co-chaperones to encounter a chaperone is crucial for chaperone activity. ERdj3, an abundant soluble endoplasmic reticulum (ER) co-chaperone of the Hsp70 BiP, stimulates the ATPase activity of BiP to increase BiP’s affinity for client (or substrate) proteins. We investigated ERdj3 availability, how ERdj3 levels impact BiP availability, and the significance of J proteins for regulating BiP binding of clients in living cells. FRAP analysis revealed that overexpressed ERdj3-sfGFP dramatically decreases BiP-GFP mobility in a client-dependent manner. By contrast, ERdj3-GFP mobility remains low regardless of client protein levels. Native gels and co-immunoprecipitations established that ERdj3 associates with a large complex including Sec61α. Translocon binding probably ensures rapid encounters between emerging nascent peptides and stimulates BiP activity in the crucial early stages of secretory protein folding. Importantly, mutant BiP exhibited significantly increased mobility when it could not interact with any ERdj3s. Thus, ERdj3 appear to play the dual roles of increasing BiP affinity for clients and regulating delivery of clients to BiP. Our data suggest that BiP engagement of clients is enhanced in ER subdomains enriched in ERdj3 proteins.

Key words: BiP, ERdj, Fluorescence recovery after photobleaching, FRAP, Quality control, Superfolder GFP

Introduction
The ability of a cell to simultaneously promote correct folding of proteins and maintain quality control (QC) of misfolded proteins is vital for cellular function. Molecular chaperones enhance folding of nascent polypeptides. To be effective, chaperones must be available to encounter nascent proteins in a brief time window. Cells can satisfy these conditions, in part, by expressing chaperones at near millimolar concentrations (Guth et al., 2004; Schwanhäusser et al., 2011; Weitzmann et al., 2007). Another factor defining chaperone availability is whether substrates can readily encounter a chaperone. Endoplasmic reticulum (ER) chaperones appear to be mobile and can diffuse to clients (i.e. substrates for ERdj3), even those clients still associated with the relatively immobile translocation channel (Blais et al., 2010; Borgese et al., 2006; Nikonov et al., 2002; Ostrovsky et al., 2009). A number of chaperone co-factors, termed co-chaperones, regulate chaperone activities (Echtenkamp and Freeman, 2012; Laufen et al., 1999; Otero et al., 2010). Many co-chaperones are expressed at substoichiometric levels relative to chaperones (Schwanhäusser et al., 2011; Weitzmann et al., 2007). To cope with varied cellular conditions, mechanisms must exist to ensure that the sufficient amounts of chaperones are available to encounter clients and co-chaperones to perform proper functions in the window of time needed to prevent client proteins from misfolding, which raises an important question. How do co-chaperones regulate chaperone function in cells?

Co-chaperones play critical roles in preventing misfolded protein stress and are often essential for cell viability (Frand and Kaiser, 1998; Jonikas et al., 2009; Kampinga and Craig, 2010). The ability of co-chaperones to encounter their dependent chaperones is likely to significantly impact the overall functionality of the global chaperone pool. While much is known regarding the biochemistry and functions of chaperones and their co-chaperones (Jin et al., 2008; Jin et al., 2009; Nakanishi et al., 2004; Shen and Hendershot, 2005), the cell biology of co-chaperones and their impact on the general availability of chaperones in cells are less well understood. It remains unclear whether co-chaperones are generally mobile and can diffuse to chaperones as needed or if co-chaperones are restricted in mobility by incorporation into large molecular complexes (Meunier et al., 2002).

Secretory proteins are translocated into the ER, where they fold and undergo posttranslational modifications (e.g. disulfide bonds and N-linked glycosylation) (Kleizen and Braakman, 2004). The constant influx of nascent secretory proteins and the crowded environment of the ER present a challenge to maintain the availability of multiple families of ER chaperones for efficient and correct folding and QC of secretory proteins (Kleizen and Braakman, 2004). Failure results in misfolded protein accumulation and activation of an adaptive stress pathway termed the unfolded protein response (UPR) to restore homeostasis (Bernales et al., 2006; Brodsky and Skach, 2011; Ron and Walter, 2007; Rutkowski and Kaufman, 2004; Walter and Ron, 2011). BiP, the ER member of the Hsp70 superfamily, is an essential and abundant ER chaperone (about 1–5 mM) (Gilchrist et al., 2006; Guth et al., 2004). BiP facilitates secretory protein folding and QC (Brodsky et al., 1995; Getling, 1999; Hendershot, 2004; Kabani et al., 2003; Kang et al., 2006) by binding exposed hydrophobic domains of unfolded proteins to prevent aggregation and help retain incompletely folded proteins in the ER (Getling, 1999; Haas, 1994). In addition, BiP helps target misfolded proteins for retrotranslocation for cytoplasmic
proteasomal degradation, a process termed ER-associated degradation (ERAD) (Brodsky and Skach, 2011; Kabani et al., 2003; Smith et al., 2011).

The many functions of BiP require its ATPase activity (Cheetham et al., 1994) to regulate binding affinity for clients, which ranges from 1 to 200 μM (Davis et al., 1999). ATP-bound BiP is in an open configuration with high on and off rates of client exchange (Marcinowski et al., 2011). ATP hydrolysis switches BiP into a closed conformation to stabilize client binding (Blond-Elguindi et al., 1993; Haas, 1994). The exchange of ADP for ATP reopens the conformation of BiP and clients then release from BiP. The early steps of the BiP ATPase chaperone cycle are regulated by J-domain containing co-chaperones (ERdj) (Awad et al., 2008; Otero et al., 2010).

Hsp40 co-chaperones enhance the ATPase activity of Hsp70 chaperones and consequently increase Hsp70 affinity for clients (Kampinga and Craig, 2010; Marcinowski et al., 2011). In mammals seven ERdj5s have been identified with widely different concentrations and affinities for BiP (Otero et al., 2010; Qiu et al., 2006). They all belong to Hsp40 family and bind to BiP via their conserved J-domains, particularly the HPD amino acid motif (Shen and Hendershot, 2005). ERdj5s possess varied sub-ER localizations and functions (Otero et al., 2010; Weitzmann et al., 2007). Some ERdj5s have been linked to the translocation of nascent peptides into the ER (Hennessy et al., 2005; Otero et al., 2010), while ERdj4 and ERdj5 are implicated in the turnover of misfolded proteins by ERAD (Dong et al., 2008; Hagiwara et al., 2011; Lai et al., 2012; Ushioda et al., 2008). ERdj3 is a ubiquitously expressed luminal glycoprotein cofactor for BiP (Shen and Hendershot, 2005; Yu et al., 2000) and binds both nascent unfolded or misfolded proteins (Jin et al., 2008; Jin et al., 2009; Shen and Hendershot, 2005). Release of clients requires association of ERdj3 with BiP (Shen and Hendershot, 2005), suggests that ERdj3 should undergo significant changes in molecular complex size, which should result in significant changes in D values (Lippincott-Schwartz et al., 2001; Snapp et al., 2003). Thus, we predicted the mobility of a fluorescently labeled ERdj3 would be as responsive to unfolded protein burdens and ER stress as BiP-GFP (Lai et al., 2010; Lajoie and Snapp, 2011). Changes in the mobility of FP-tagged ERdj3, should report on interactions with clients and BiP. Subsequent increases or decreases in size are measured by changes in D values. An increase in co-chaperone complex size by a factor of two would decrease D by half, resulting in a decreased sampling area and less access or availability of the molecule to chaperones or clients in the ER. Thus, even modest changes in D values can reflect biologically significant changes (Lai et al., 2010).

Recent live cell photobleaching analysis by our lab revealed that BiP, tagged with a fluorescent protein (FP), is mobile and can readily sample the entire ER to encounter client proteins (Lai et al., 2010). The mobility of BiP is highly dependent on client protein levels. Translational inhibition increases BiP mobility, while unfolded protein accumulation significantly decreases the diffusion of BiP. Thus, BiP-GFP is a powerful tool for measuring changes in the global pool of unfolded secretory proteins, as well as overall BiP occupancy with clients. To efficiently facilitate BiP function, ERdj5s must encounter or recruit BiP and client substrates. In this study we investigated the availability of ERdj3 in live cells and its impact on regulation of BiP availability. By imaging fluorescent protein tagged ERdj3 and BiP in live cells we investigated: 1) How available is ERdj3 within the ER? 2) What factors regulate ERdj3 availability? and 3) How much do ERdj5s impact BiP availability in cells?

**Results**

**Rationale and experimental approach**

Our goal was to investigate the cell biology of BiP co-chaperones and their regulation of BiP function and availability in intact cells. Although there are seven human ERdj5s (Qiu et al., 2006), several of the ERdj5s are not suitable for photobleaching studies. ERdj1 and ERdj2 are ER membrane proteins, which are unlikely to be responsive to changes in complex size, because the viscosity of the ER membrane dominates the effects of molecular size changes (Marguet et al., 1999; Saffman and Delbrück, 1975; Snapp et al., 2003). We focused on one of the soluble ERdj5s, ERdj3.

To quantify changes in BiP and ERdj3 mobility, we combined fluorescent protein fusions and photobleaching techniques, especially Fluorescence recovery after photobleaching (FRAP) (Lippincott-Schwartz et al., 2001; Wouters et al., 2001). In this technique, a region of interest (ROIs) is scanned with high intensity laser light to destroy the fluorescence of FP-tagged fusion proteins in the ROI. Mobile unbleached molecules then diffuse into the ROI and the increase in fluorescence intensity can be quantified. The mobility or availability changes of a protein are reflected by the effective diffusion coefficient (D), which quantitates both changes in the environmental viscosity and the size of a molecule or an associated complex (Snapp et al., 2003). The ability of ERdj3 to bind and release unfolded client proteins, as well as associating with BiP (Shen and Hendershot, 2005), suggests that ERdj3 should undergo significant changes in molecular complex size, which should result in significant changes in D values (Lippincott-Schwartz et al., 2001; Snapp et al., 2003). Thus, we predicted the mobility of a fluorescently labeled ERdj3 would be as responsive to unfolded protein burdens and ER stress as BiP-GFP (Lai et al., 2010; Lajoie and Snapp, 2011). Changes in the mobility of FP-tagged ERdj3, should report on interactions with clients and BiP. Subsequent increases or decreases in size are measured by changes in D values. An increase in co-chaperone complex size by a factor of two would decrease D by half, resulting in a decreased sampling area and less access or availability of the molecule to chaperones or clients in the ER. Thus, even modest changes in D values can reflect biologically significant changes (Lai et al., 2010). This approach has been successfully applied to study other ER chaperones, including calreticulin, GRP94, and BiP (Lai et al., 2010; Lai et al., 2012; Lajoie et al., 2012; Ostrovsky et al., 2009; Snapp et al., 2006). In addition, an inert probe, ER-RFP that does not interact with other proteins in the ER lumen, is used to report on changes in ER crowdedness (Dayel et al., 1999; Lai et al., 2010; Snapp et al., 2006).

**ERdj3-sfGFP is functional**

We engineered ERdj3-sfGFP (Fig. 1A) by fusing the fluorescent tag superfolder GFP (sfGFP) (Aronson et al., 2011; Pédelaq et al., 2006) to the C-terminus of human ERdj3. When expressed in cells, our construct correctly colocalizes in a tubular network pattern with ER-RFP, consistent with previous reports of ER localization (Fig. 1B) (Nakanishi et al., 2004; Yu et al., 2000). In an immunoblot, ERdj3-sfGFP migrates more slowly (70 kDa) than endogenous ERdj3 (~40 kDa), consistent with the addition
of the 25 kDa sfGFP (Fig. 1C). In transiently transfected cells, the expression level of ERdj3-sfGFP was approximately twofold higher than endogenous ERdj3. In addition, we confirmed that ERdj3-sfGFP is N-linked glycosylated at N261 in the ER, as wild type ERdj3 is. Cells expressing ERdj3-sfGFP were treated with tunicamycin (Tm), an inhibitor of N-linked glycosylation, and then lysed. In immunoblots probed with either anti-ERdj3 or anti-GFP, we found a fraction of endogenous ERdj3 treated with Tm migrated faster than the protein from untreated cells (Fig. 1C) consistent with glycosylation. A similar band shift was observed for ERdj3-sfGFP in lysates treated with Tm. Thus, ERdj3-sfGFP appears to be correctly posttranslationally processed.

To establish the functionality of the ERdj3-sfGFP fusion, we first tested whether the construct could interact with endogenous BiP (Fig. 2A). Cells transfected with either wild-type ERdj3-sfGFP or a mutant unable to bind BiP, H53Q ERdj3-sfGFP (Shen and Hendershot, 2005), were lysed and then co-immunoprecipitated with anti-GFP beads. The bound proteins were probed in immunoblots with anti-ERdj3. Bound BiP was only detected when lysates contained wild-type ERdj3-sfGFP, while no bound endogenous BiP was observed in either untransfected or cell lysates (Fig. 2A).

Furthermore, we tested the ability of ERdj3-sfGFP to form oligomers. Previous studies established that ERdj3 forms homo-oligomers and oligomerization is required for ERdj3 function (Jin et al., 2009). As F326 near the C-terminal of ERdj3 is critical for oligomerization (Jin et al., 2009), we used this mutant as a negative control to determine whether the fusion of sfGFP to the C-terminal of ERdj3 impacts self-assembly. Cells coexpressing ERdj3-mCherry and ERdj3-sfGFP or ER-sfGFP were lysed, co-immunoprecipitated with anti-GFP, and the bound proteins were probed in immunoblots with anti-RFP. ERdj3-mCherry was only pulled down in cell lysates containing wt ERdj3-sfGFP (Fig. 2B,C), whereas F326D mutant ERdj3-sfGFP failed to pull down with ERdj3-mCherry (Fig. 2C). Thus, the C-terminal fusions of FPs to ERdj3 do not perturb oligomerization. Taken together, our ERdj3-sfGFP retains properties of native ERdj3 and should be suitable for live cell imaging and mobility assays.

ERdj3 availability in the homeostatic ER

Previously, our lab reported that the majority of BiP diffuses homogenously throughout the ER (Lai et al., 2010). We asked whether ERdj3 is mobile in the ER lumen in unstressed cells. To address this question we performed Fluorescence loss in photobleaching (FLIP) (Ellenberg and Lippincott-Schwartz, 1997), which resembles FRAP except the ROI is alternatingly imaged and photobleached multiple times. If molecules are mobile within a continuous compartment that passes through the ROI, fluorescence will be depleted from that compartment (Ellenberg and Lippincott-Schwartz, 1997; Lai et al., 2010). However, immobilized molecules or molecules in a discontinuous compartment will remain unbleached. We performed dual-color FLIP on a single cell coexpressing ERdj3-sfGFP and ER-RFP (Fig. 3). Both ER-RFP and ERdj3-sfGFP fluorescence were homogenously depleted from the entire ER (Fig. 3, upper panel). Thus, the majority of ERdj3 is mobile throughout the ER.

However, the rate of fluorescence depletion of ERdj3-sfGFP was much slower than the slightly smaller ER-RFP (Fig. 3). This could be related to formation of oligomers, binding to clients, and/or incorporation into a larger complex. To quantitate how mobile ERdj3-sfGFP is in cells, we performed FRAP analysis. Dual-color FRAP of ERdj3-sfGFP and ER-RFP confirmed the FLIP result. ER-RFP fluorescence recovered much more quickly than ERdj3-sfGFP (Fig. 4A). Quantitative FRAP of ERdj3-sfGFP relative to BiP-mGFP and inert ER-sfGFP (Fig. 4B) revealed that the larger BiP-GFP diffused significantly more slowly than the smaller ER-sfGFP ($D_{\text{BiP-GFP}}=0.36\pm0.05$ vs $4.2\pm0.2$) (Lai et al., 2010). However, ERdj3-sfGFP, which is smaller than BiP-GFP and the ERdj3 dimer is not much larger, would be predicted to diffuse faster than BiP-GFP. Instead, ERdj3-sfGFP was much less mobile ($D_{\text{ERdj3-sfGFP}}=0.12\pm0.01$) (Fig. 4B).

$D$ values can be used to infer something about the size of a molecule. Using the Stokes-Einstein relationship, where $D$ is inversely proportional to environmental crowdedness multiplied by the hydrodynamic radius, $R_h$ (Einstein, 1905), we estimated the $R_h$ of ERdj3-sfGFP was $\sim80$ nm, based on its $D$ value.
FRAP of ER-RFP in the absence and presence of ERdj3-sfGFP in MDCK cells (supplementary material Fig. S1A) revealed no statistically significant difference in ER-RFP mobility. We also considered whether overexpression of ERdj3-sfGFP might impact its mobility. Perhaps higher levels of ERdj3-sfGFP would cause higher order oligomerization. If so, higher expressing cells should exhibit the lowest \( D \) values. No such correlation was observed (supplementary material Fig. S1B). Therefore, we hypothesized that ERdj3 associates with a large, possibly membrane bound complex.

**ERdj3 mobility is independent of oligomerization, binding clients or BiP**

To determine what properties of ERdj3 might govern association with a complex, we investigated the mobilities of various ERdj3 mutants. We first tested whether BiP binding regulates ERdj3’s low mobility. FRAP analysis of wildtype and the BiP binding mutant (Shen and Hendershot, 2005) H53Q ERdj3-sfGFP (Fig. 5A) revealed that inhibiting BiP binding significantly increases ERdj3-sfGFP mobility (0.2±0.01 \( \mu \text{m}^2/\text{second} \) versus 0.12±0.01) (Fig. 5A). However, the mean \( D \) value of H53Q ERdj3-sfGFP is still much lower than we would anticipate for a soluble luminal protein. For example, even the 210 kDa dimer GRP94-mGFP diffuses slightly faster than ERdj3-sfGFP (Ostrovsky et al., 2009). Thus, association with BiP contributes to low ERdj3-sfGFP mobility, but is not the primary regulator of ERdj3-sfGFP diffusion in the ER.

We then asked whether ERdj3 oligomerization (Jin et al., 2009) contributes to the low diffusion. We predicted \( D \) would increase in the absence of oligomerization. Yet, \( D \) values of the F326D nonoligomerizing mutant were comparable to wt ERdj3-sfGFP (0.11±0.02 \( \mu \text{m}^2/\text{second} \)) (Fig. 5A).

Previously, our lab demonstrated the lower than predicted mobility BiP-GFP is primarily due to interactions with clients (Lai et al., 2010). ERdj3 can bind similar clients (Marcinowski et al., 2011; Shen and Hendershot, 2005) and this should decrease ERdj3 mobility. Nascent client proteins were depleted from the ER by treating cells with the translational inhibitor pactamycin (Pact). As observed previously, Pact treatment increased the mean \( D \) value of the control BiP-GFP ~threefold (0.5±0.04 \( \mu \text{m}^2/\text{second} \) versus 1.4±0.1 \( \mu \text{m}^2/\text{second} \)), approximating the predicted \( D \) value for freely diffusing BiP-GFP (1.6 \( \mu \text{m}^2/\text{second} \)) (Fig. 5B). In contrast, no significant increase in \( D \) for ERdj3-sfGFP (0.24±0.03 \( \mu \text{m}^2/\text{second} \) versus 0.29±0.04 \( \mu \text{m}^2/\text{second} \)) was observed (Fig. 5B) indicating client levels do not appear to impact ERdj3 mobility. We further investigated this possibility by introducing the V153A mutation, which impairs ERdj3 interactions with clients (Shen and Hendershot, 2005). FRAP analysis revealed the mutation had no effect on ERdj3 mobility (Fig. 5C). A potential confounder was that the V153A mutant could still hetero-oligomerize with endogenous ERdj3, which should still bind clients. The double mutant V153A/F326D did not significantly change \( D \) values relative to wild type ERdj3 either (0.11±0.01 \( \mu \text{m}^2/\text{second} \) versus 0.13±0.02 \( \mu \text{m}^2/\text{second} \)) (Fig. 5C). Thus, client binding does not affect ERdj3 mobility in cells.

**ERdj3-sfGFP associates with a complex containing Sec61α**

Our FRAP data suggested that ERdj3-sfGFP associates with a large molecular complex. To investigate the possibility of ERdj3 complexes, we performed native gel electrophoresis for endogenous ERdj3 and transiently expressed ERdj3-sfGFP. In

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(0.12 \( \mu \text{m}^2/\text{second} \)) relative to the 2.3 nm \( R_b \) and \( D \) value of ER-sfGFP. However, this estimate is unlikely to be correct, as it is larger than the diameter of ER tubules (40–70 nm) (Hu et al., 2011; Snapp, 2003). Instead, the low \( D \) value likely reflects binding and release of ERdj3 from exceptionally large or even immobile molecular complexes, which could lower the mobility of a molecule regardless of its actual size.

One caveat for interpreting the experiment is that overexpression of ERdj3-sfGFP (Fig. 1C) could somehow increase ER viscosity and decrease ERdj3 mobility. However,
Fig. 3. ERdj3-sfGFP is mobile throughout the ER. U2OS cells, transiently co-transfected with ERdj3-sfGFP and ER-RFP, were subjected to dual color FLIP. Both ERdj3-sfGFP and ER-RFP fluorescence were homogenously depleted from the entire ER (upper panel). The graph below shows the rate of fluorescence depletion. ERdj3-sfGFP (green dotted line) fluorescence was depleted, but much more slowly than ER-RFP (red dotted line). Fluorescence loss was specific, as adjacent cells remained fluorescent (solid green and red lines). Scale bar: 10 μm.

Fig. 4. Quantification of ERdj3-sfGFP mobility in the ER. (A) FRAP analysis of transiently transfected MDCK cells coexpressing ER-RFP and ERdj3-sfGFP. Images of cells after photobleaching the ROI (boxed area) reveal that ER-RFP recovers much more rapidly than ERdj3-sfGFP. Scale bar: 10 μm. (B) Representative fluorescence recovery plots illustrate the dramatically faster diffusion of ER-RFP relative to ERdj3-sfGFP. (C) Quantification reveals that ERdj3-sfGFP diffuses much more slowly than BiP-mGFP and ER-sfGFP. FRAP of MDCK cells transiently transfected with ERdj3-sfGFP, ER-sfGFP and BiP-mGFP. D values for single cells are plotted; horizontal lines indicate mean D values; P values are given above the columns.
Fig. 5. The low mobility of ERdj3-sfGFP is independent of BiP binding, client levels and oligomerization. (A) Transiently transfected MDCK cells expressing ERdj3-sfGFP or the H53Q or F326D ERdj3-sfGFP mutants were analyzed by FRAP. (B) MDCK cells transiently transfected with ERdj3-sfGFP were treated with 0.2 μM Pact for 1 hour and analyzed by FRAP. (C) Transiently transfected MDCK cells expressing ERdj3-sfGFP, F326D ERdj3-sfGFP or V153A/F326D ERdj3-sfGFP mutants were analyzed by FRAP. D values for single cells are plotted; horizontal lines indicate mean D values; P values are given above the columns; error bars indicate s.e.m.

the native gel immunoblot probed with anti-ERdj3, the majority of endogenous ERdj3 migrated exceptionally slowly at ~220 kDa (Fig. 6A). Although ERdj3 is ~40 kDa, no ERdj3 was detected at that range or even as an 80 kDa dimer (Fig. 6A). Similarly, transiently transfected ERdj3-sfGFP also migrated at a high molecular weight (Fig. 6A), even at low expression levels during early times post-transfection. Such a complex would be anticipated to significantly impact the mobility of ERdj3.

What might be in the ERdj3 complex? We considered two previously described complexes, a chaperone complex and the Sec61 translocon. Cross-linking studies have described a ~400 kDa ER multi-chaperone complex including BiP, GRP94, PDI, and ERdj3 (Meunier et al., 2002). We compared the electrophoretic mobility of the ERdj3 complex with the abundant ER chaperones reported for this complex. However, we did not observe the co-migration of GRP94 or PDI relative to the ERdj3 complex in the native gel electrophoresis (Fig. 6B). Thus, these other ER chaperones do not appear to be a major component of the large ERdj3 complex. While our data suggest only a relatively small subpopulation of ERdj3 could incorporate in multi-chaperone complexes in MDCK cells, this result could vary between cell types or may depend on extraction conditions.

Another possibility was the recently reported association between ERdj3 and the Sec61 translocon (Dejgaard et al., 2010). In mitochondria, J proteins have been reported to associate with translocation machinery to assist with the import of proteins (Pais et al., 2011). In the ER, ERdj1 (Mtj1) and ERdj2 (Sec63) associate with the Sec61 translocon complex (Alder et al., 2005; Brodsky et al., 1995; Dudek et al., 2005; Meyer et al., 2000) and can stimulate BiP interactions with nascent clients during translocation into the ER (Alder et al., 2005; Brodsky et al., 1995; Dudek et al., 2005; Otero et al., 2010). Association of ERdj3-sfGFP with the very slowly diffusing Sec61 translocon (Nikonov et al., 2002) could readily account for its extremely low mobility. We investigated whether ERdj3-sfGFP associates with the Sec61 translocon by performing anti-GFP co-immunoprecipitation of lysates from cells transfected with ERdj3-sfGFP. Bound proteins were probed in immunoblots with anti-Sec61α, a component of the Sec61 translocon complex (Fig. 6C). We detected Sec61α when lysates contained ERdj3-sfGFP, consistent with their association, though it remains unclear if this interaction is direct. As Sec61α is highly abundant, the result is consistent with our system in that even overexpressing ERdj3 is unlikely to easily saturate the Sec61α binding sites. We never observed an increase in ERdj3 mobility in highly expressing cells, suggesting that there are abundant binding sites for ERdj3. In future studies, we are seeking to identify the necessary sequence motifs for ERdj3-Sec61α complex interactions. We consider the implications of Sec61α association in the Discussion below.
Overexpression of ERdj3 decreases BiP mobility

As ERdj3 interacts with BiP and appears to diffuse more slowly than BiP, we asked whether overexpression of ERdj3 decreases BiP mobility. BiP-mCherry was transfected into MDCK cells in the absence and presence of wild type or the BiP binding mutant (H53Q) ERdj3-sfGFP and $D$ values were analyzed by dual-color FRAP (Fig. 7A). Coexpression of BiP-mCherry did not alter the mobility of wild type or mutant ERdj3-sfGFP in cells (supplementary material Fig. S1C). In contrast, BiP-mCherry mobility significantly decreased in the presence of ERdj3-sfGFP (0.5±0.08 μm²/second versus 0.1±0.02 μm²/second) (Fig. 7A). The decrease in $D$ values of BiP-mCherry depended on its interaction with ERdj3-sfGFP, as the binding mutant H53Q ERdj3-sfGFP had no effect on the availability of BiP-mCherry (0.5±0.03 μm²/second) (Fig. 7A). Thus, ERdj3 levels can significantly regulate BiP mobility. Interestingly, the apparent increased occupancy of BiP in ERdj3-sfGFP transfected cells did not detectably activate the UPR (supplementary material Fig. S2).

How might ERdj3 decrease BiP mobility? We considered two potential hypotheses. First, Sec61a-bound ERdj3 could simultaneously bind BiP and, thus, lower BiP mobility. Another possibility is that high levels of ERdj3 increase client loading of BiP, increasing the percentage of client-bound BiP (Jin et al., 2009; Shen and Hendershot, 2005).

ERdj3s significantly regulate BiP–client binding in cells

First, we tested whether association with ERdj proteins in general significantly regulates BiP mobility. We asked whether interactions between BiP and the entire pool of ERdj3s impact BiP availability and, thus, function in cells. We transiently transfected MDCK cells with R197H mutant BiP-GFP, which disrupts BiP binding to J-domains, but not endogenous BiP ATPase activity (Alder et al., 2005; Awad et al., 2008; Vembar et al., 2010). FRAP of the wt and mutant BiP-mGFP revealed a significant difference in mobilities. In the absence of ERdj binding, BiP mobility was significantly higher than wt (0.5±0.04 μm²/second versus 0.9±0.1 μm²/second) (Fig. 7B). Even more dramatically, when misfolded secretory proteins accumulated with Tm treatment, the mobility of wild type BiP-GFP decreased significantly, consistent with our finding that misfolded proteins slow BiP diffusion (Lai et al., 2010), while R197H BiP-GFP did not (0.25±0.02 μm²/second versus 0.7±0.1 μm²/second) (Fig. 7B). Binding of ERdj3s stimulates BiP ATPase activity (Marcinowski et al., 2011), primes ADP-bound BiP for interactions with proteins and slows the rate of client release upon exposure to ATP. Thus, disruption of J-domain association with BiP appears to significantly lower its affinity for the global ER client burden. Therefore, in cells, BiP must depend on interactions with J proteins to associate with most clients.

Next, we tested whether ERdj3 overexpression can impact BiP mobility in the absence of clients or the ability to bind clients. Wt or mutant ERdj3-mCherry was co-transfected with wt BiP-mGFP or a client-binding BiP mutant [T453D, similar to the Kar2p substrate-binding mutant kar2-133 (Kabani et al., 2003)]. While wt BiP mobility specifically decreases with overexpressed ERdj3 (0.7±0.1 μm²/second versus 0.2±0.02 μm²/second), the client-binding mutant diffuses more rapidly than wt BiP (1.4±0.2 μm²/second) and is unaffected by wt (1.3±0.1 μm²/second) or mutant ERdj3 (1.7±0.2 μm²/second) expression (Fig. 7C). This result strongly argues against a model in which ERdj3 directly tethers BiP to the Sec61 translocon. It is possible that the T453D mutant could have a decreased affinity for ERdj3. However, co-IPs revealed similar levels of binding of T453D BiP to ERdj3 (Fig. 7D). In a complementary experiment, we found that depletion of clients with Pact treatment partially restored BiP mobility in the presence of overexpressed ERdj3 (0.1±0.02 μm²/second versus 0.3±0.04 μm²/second) (Fig. 7E). Taken together,
the ability of ERdj3 or ERdj proteins in general, to decrease BiP mobility depends on the ability of ERdj3 to load BiP with clients.

**Discussion**

Co-chaperones regulate and stimulate functions of partner chaperones. The availability of each ERdj is likely to impact BiP activity and function. Previously, our lab reported that BiP is highly mobile in the ER lumen (Lai et al., 2010) and we postulated that ERdj3 would be similarly mobile. Since ERdj3 appears to assist BiP in folding and QC of nascent peptides (Jin et al., 2009; Nakamichi et al., 2004; Shen and Hendershot, 2005), ERdj3 must be able to encounter clients and BiP in the ER. A sufficient concentration and mobility of ERdj3 would ensure efficient encounters with clients and BiP. However, our live cell photobleaching and native gel electrophoresis results revealed the mobilities of fluorescently tagged or endogenous ERdj3 were extraordinarily low. The pactamycin and substrate-binding ERdj3 mutant results strongly suggest that the low ERdj3 mobility is independent of interactions with clients. In contrast, the low mobility is consistent with association between ERdj3 and a large complex, potentially the Sec61\alpha translocon. The extremely large Sec61 translocon (Beckmann et al., 2001; Hanein et al., 1996; Menetret et al., 2000) diffuses quite slowly, especially when assembled with polysomes (Nikonov et al., 2002). Furthermore, there appear to be a sufficient number of Sec61\alpha binding partners for the ERdj3 pool in cells. Schwanhausser et al. (Schwanhäuser et al., 2011) estimated a typical tissue culture cell contains ~200,000 copies of Sec61\alpha and ~58,000 copies of ERdj3. If each translocon includes at least two copies of Sec61\alpha (Snapp et al., 2004), then most translocons should have an associated ERdj3 and each ERdj3 should be translocon-bound at steady state. Our FRAP data of wt and mutant ERdj3s are consistent with this interpretation. In addition, we typically examined ERdj3-sfGFP expressing cells with only modest increases in total ERdj3 levels (i.e. less than twofold), below a saturating threshold (supplementary material Fig. S3). In this range, we did not observe and differences in D values relative to low to intermediate expression levels of ERdj3-sfGFP (supplementary material Fig. S1B).

The functional consequences of ERdj3 association with the translocon could help explain ER retention of ERdj3, as the co-chaperone lacks a KDEL sequence. In addition, BiP is reported to interact with the metazoan Sec61 translocon and play a role in gating the translocon to maintain luminal ER calcium levels (Hamman et al., 1998). BiP-mediated translocon pore closure and opening requires functional interactions between both the substrate-binding region and the J domain-binding region of BiP (Aldere et al., 2005). ERdj3 could act as a localized regulator of BiP’s gating activity. Other ERdj3 have reported roles in mediating protein translation and translocation, including ERdj1/ Mti1p that interacts with translating ribosomes and may be involved in regulation of protein translation (Dudek et al., 2005), ERdj7, a newly identified resident ER protein with unknown function (Zahedi et al., 2009), and ERdj2/Sec63 that associates with Sec61 translocon (Brodsky et al., 1995; Meyer et al., 2000). ERdj3 appears to represent another localized regulator of BiP activity with nascent peptides at the translocon.

At this time, we cannot rule out interactions between ERdj3 and a translocon-independent pool of Sec61\alpha. However, the vast majority of Sec61\alpha is incorporated in ribosome-bound complexes in mammalian cells (Meyer et al., 2000).

UPR-stimulated upregulation of ERdj3 and the ability of ERdj3 to bind misfolded proteins suggest ERdj3’s primary roles are protein folding and QC (Shen and Hendershot, 2005). Binding of ERdj3 to the translocon provides an elegant mechanism by which ERdj3 efficiently and preferentially encounters newly synthesized peptides as they are translocated through the Sec61 translocon, regardless of the presence of BiP (Shen and Hendershot, 2005). Binding of nascent peptide clients to ERdj3 associated with the translocon would quickly protect the unfolded clients from aggregation. Sec61 translocon associated ERdj3 could transfer clients to freely diffusing unoccupied BiP molecules in the close proximity of the translocon.

The exceptionally low ERdj3 diffusion coefficient makes ERdj3 relatively unavailable to proteins that have been released from and diffused away from the translocon. However, other ERdjs, ERdj4 and ERdj5, have been implicated in the recognition and retrotranslocation of misfolded secretory proteins during ERAD (Dong et al., 2008; Kurisu et al., 2003; Pröls et al., 2001; Shen et al., 2002). Lai et al. recently reported that ERdj4 associates with the ERAD component Derlin1 (Lai et al., 2012). ERdj3- and ERdj4-specific interactions with distinct pools of clients could be spatially restricted to where the ERdj3s localize. Alternatively, and not mutually exclusive is the possibility that ERdj3 may be spatially restricted to nascent clients, while ERdj4 freely diffuses (Lai et al., 2012), binds, and shepherds ERAD clients to the retrotranslocon.

While our data do not preclude the possibility that BiP, in cells, may directly bind some nascent clients in the absence of ERdj proteins, the BiP mutant data suggest that BiP occupancy is highly dependent on interaction with ERdj proteins during homeostasis and stress. Blocking interaction between BiP and J-domain of all ERdj is significantly decrease BiP affinity to clients in vivo. Our FRAP results in Fig. 7 suggest how ERdj3 and its yeast homolog, Scj1p, could play a major role in ER homeostasis. Ascj1 yeast are viable, but grow slowly and exhibit constitutive UPR activation (Jonkas et al., 2009). A decreased population of available ERdj proteins (note that Scj1p is the most abundant of five yeast ERdj proteins) could decrease the ability of BiP to tightly bind clients and thus decrease BiP function, regardless of how much BiP levels are upregulated in cells. In future studies, we will explore the relative contributions of the different ERdj proteins to BiP interactions with the global pool of clients. An interesting prediction of our findings is that overexpression of ERdj3 will slow the rate of secretion of nascent proteins from the ER.

Finally, we were intrigued by the observation that ERdj3 overexpression slowed BiP mobility in a client-dependent manner, similar to the decreased BiP mobility observed in cells treated with UPR-inducing stresses including DTT and Tm. Yet, overexpressed ERdj3 did not result in obvious UPR activation. Even though ERdj3 is upregulated during the UPR, overexpression of ERdj3, alone did not confer resistance to the Tm-induced or thapsigargin UPR (Nakanishi et al., 2004). Occupancy of BiP is thought to buffer cells against increasing levels of unfolded proteins (Pincus et al., 2010). Saturated BiP should increase the probability that the UPR sensors IRE1 and PERK will encounter unfolded peptides, leading to UPR activation (Gardner and Walter, 2011; Onn and Ron, 2010). However, ERdj3 overexpression does not appear to increase production of unfoldable secretory proteins typical of damaged proteins generated by DTT or Tm treatment. Therefore, increased
BiP occupancy may be less important than increased levels of clients that can potentially bind UPR sensors. Future ERdj3 overexpression studies may help provide new insights into the relationship between BiP occupancy, client levels, and the types of clients necessary for UPR activation in metazoan cells.

Materials and Methods

Drugs

Pactamycin (a gift from E. Steinbrecher, Pharmacia Corp., Peapack, NJ) was dissolved in distilled H2O in a 20 μM stock (Snapp et al., 2004) and used at 0.2 μM for 1 hour. Tunicamycin (Tm) (Calbiochem, La Jolla, CA) was dissolved in DMSO at a 5 mg/ml stock and used at the concentrations and times indicated.

Plasmid constructs

Human ERdj3-EGFP was provided by Linda Hendershot (St. Jude Children’s Research Hospital, Memphis, TN). ERdj3 cDNA was subcloned into EcoRI and BamHi sites of sfGFP (Aronson et al., 2011) or mCherry (Shaner et al., 2004) vector based on the Clontech N1-GFP backbone (Pal Alto, CA). ERdj3 mutants were made by site-directed mutagenesis of ERdj3-sfGFP or ERdj3-mCherry serving as the templates. ERdj3 mutants were generated by PCR using the following primer pairs. H53Q, forward, 5’-CTGCAACCTGCAGCCCGACGAGATACCA-3’, reverse, 5’-CTGCGCTGCCGCGTAGAAGATC-3’, forward, 5’-CTTTTGATGTGATGACCAAAAGACG-3’, reverse, 5’-CTTTTTGTGACGTATCAAGAAAAG-3’, V153A, forward, 5’-GAAATTGTTGTGAGCCGTTTACAAAAC-3’, reverse, 5’-GAGTGTGGTTTCTAAAGCGTCGTCAAATTT-3’. ER-sfGFP, ER-red fluorescent protein (RFP) and hamster BiP-mGFP have been previously described (Lai et al., 2010; Snapp et al., 2006). BiP mutants were generated by PCR using the following primer pairs. R197H, forward, 5’-CTGAAATTGTGCCCATCATTCAATGAGC-3’, reverse, 5’-GCGCTGTTAGACGAGGAGTTCA-3’, E539R, forward, 5’-CAATTTCCCATCCTTTGAATTCTGTTGACG-3’, reverse, 5’-CTAAATGATATGAGTTGTAATTCTG-3’. Mutant constructs were confirmed by sequencing. All constructs were transiently transfected for 16–48 hours into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Cells

Madin–Darby canine kidney (MDCK) and U2-OS cells were grown in RPMI plus L-glutamine, 10% heat-inactivated fetal bovine serum, and penicillin/streptomycin at 37°C in 5% CO2. For all imaging experiments, cells were grown in eight-well LabTek coverglass chambers (Nunc, Naperville, IL).

Immunofluorescence and imaging of live and fixed cells

Cells were imaged in phenol-red free RPMI supplemented with 10 mM HEPES, L-glutamine, and 10% fetal bovine serum. Live cells were imaged on a 37°C environmentally controlled chamber of a confocal microscope system (Duoscan; Carl Zeiss MicroImaging, Thornwood, NY) with a 63x/1.4 NA oil objective and a 489 nm 100 mW diode laser with a 500–550 nm bandpass filter for GFP and a 40 mW 561 nm diode laser with a 565 nm longpass filter for mCherry or mRFP. Composite figures were prepared using Photoshop CS4 and Illustrator CS4 software (Adobe Systems, San Jose, CA).

Photo bleaching analysis

Fluorescence Recovery after Photobleaching (FRAP) and Fluorescence Loss in Photobleaching (FLIP) were performed by photobleaching a small region of interest (ROI) with full laser power (3 iterations, scan speed 6 for FRAP or 1 iteration scan speed 9 for FLIP) and monitoring fluorescence recovery or loss over time at low laser power (1% transmission) (Sigga et al., 2000; Snapp et al., 2003). Images were acquired with an open pinhole. No photobleaching of the cell or adjacent cells during fluorescence recovery was observed. Fluorescence recovery plots and diffusion D measurements were obtained by photobleaching a 100x wide strip and calculated using an inhomogenous diffusion simulation (Sigga et al., 2000; Snapp et al., 2003). P values were calculated using a Student’s two-tailed t test in Prism 5.0 (GraphPad Software, San Diego, CA). The relatively large spread of D values for ER proteins likely reflects differences in ER geometry between cells (Sthalzarin et al., 2005). For this reason, we use the more stringent significance cutoff of P < 0.01 to define differences in distributions as statistically significant in FRAP analyses. To create the fluorescence recovery curves, the fluorescence intensities were transformed into a 0–100% scale in which the prebleach point time equals 100% fluorescence intensity. Image analysis was performed using NIH Image 1.62 and LSM image examiner software. Fluorescence recovery curves were plotted using Prism software.

Immunoblot and co-immunoprecipitation

Total cell lysates for immunoblotting were prepared in 1% SDS, 0.1 M Tris-HCl, pH 8.0, using cells at 80–90% confluence. Proteins were separated using 7.5% or 12% Tricine SDS gels, transferred to nitrocellulose, probed with the indicated antibodies, and developed using enhanced chemiluminescence reagents from Pierce (Rockford, IL) and exposed to X-ray film. Antibodies used included rabbit anti-GFP and anti-RFP (gifts from Ramanuj. S. Hegde, Laboratory of Molecular Biology, Cambridge, UK), anti-GRP78 (BD Biosciences, San Jose, CA; mouse 610979 or Sigma-Aldrich ET-21), anti-ERdj3 (Santa Cruz, CA; sc-271240 c-7), anti-Sec61α (Affinity BioReagents, CO; PA-3104), horseradish peroxidase-labeled anti-rabbit and anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA). For co-immunoprecipitation analyses, cells in six-well plates were washed twice with 1× PBS and lysed with either NP-40 lysing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and 0.5% NP-40) for ERdj3-Sec61α interaction assays or Triton lysing buffer (1% Triton X-100, 50 mM HEPES, pH 7.4, 100 mM NaCl) for other assays. Lysis buffers contained EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN). Lysates were clarified for 10 minutes at maximum speed in a microcentrifuge at 4°C and incubated for at least 2 hours at 4°C with either anti-GFP agarose beads (MBL, Woburn, MA) or protein A agarose beads (Bio Rad Laboratories, Hercules, CA) plus the indicated primary antibody. Beads were washed four times in IP lysing buffer and once in distilled water, eluted in SDS-PAGE sample buffer, and analyzed on 7.5% or 12% SDS tricine minigels, followed by blotting, staining, and development as for standard immunobots.

Native gel electrophoresis

The native gel electrophoresis and sample preparation were modified from the protocol in (Freiden et al., 1992). Cells 80–90% confluent in six-well plates were washed twice with ice-cold cell wash buffer (20 mM HEPES pH 7.4, 150 mM NaCl) and lysed with cell lysis buffer (0.2% Triton X-100, 20 mM HEPES pH 7.4, 150 mM) containing EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN). Lysates were clarified for 10 minutes at maximum speed in a microcentrifuge at 4°C, mixed with sample buffer (250 mM Tris-HCl pH 6.8, 1% Triton X-100, 45% glycerol, 0.12% Bromphenol Blue) and analyzed on 7.5% Tricine gel without SDS at 4°C, followed by blotting, staining and development as for standard immunobots.

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Author contributions

F.G. performed the experiments; E.L.S. and F.G. designed the experiments, interpreted the results, and wrote the manuscript.

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References


**Fig. S1.** (A) Overexpression of ERdj3-sfGFP does not perturb luminal ER organization. FRAP analysis of transiently transfected MDCK cells expressing ERRFP alone or co-expressing ERdj3-sfGFP (+ERdj3-sfGFP). D values for individual cells are plotted. No significant differences were detected. (B) ERdj3-sfGFP mobility is independent of its expression level. Gain settings (higher indicates dimmer lower expressing cells) used to acquire FRAP images of each cell are plotted relative to D values of ERdj3-sfGFP. No correlation was observed. (C) Expression of BiP-mCherry has no affect on ERdj3-sfGFP mobility. Transiently transfected MDCK cells co-expressing BiP-mCherry and wt ERdj3-sfGFP or the BiP binding mutant H53Q ERdj3-sfGFP were analyzed by FRAP. D values of ERdj3-sfGFP in individual cells are plotted. The expression of BiP-mCherry does not significantly affect wt or H53Q ERdj3-sfGFP mobility.

**Fig. S2.** The decrease in BiP mobility caused by ERdj3 overexpression does not lead to detectable ER stress. (A) Transiently transfected MDCK cells expressing wt or the H53Q ERdj3-sfGFP, ER-sfGFP or Untransfected (Un) plus or minus 0.1 µg/ml Tm O/N were lysed, separated by SDS-PAGE, and immunoblotted for UPR activation with anti-BiP or the loading control anti-α-tubulin or for the GFP sfGFP construct. Increased BiP expression is only observed in the Tm treated cells. (B) Transiently transfected MDCK cells expressing sfGFP, ERdj3-sfGFP or H53Q ERdj3-sfGFP were untreated or treated with 0.1 µg/ml of Tm for 16 h, fixed and stained for the UPR marker CHOP and anti-mouse Alexa 555. Increased levels of nuclear CHOP were only observed in cells treated with Tm. Scale bar = 10 µM

**Fig. S3.** ERdj3-sfGFP expression is variable in transiently transfected cells. MDCK cells untransfected or 18 h post-transfection with ERdj3-sfGFP were fixed and stained by immunofluorescence with anti-ERdj3 and Alexa 555 anti-mouse. Images were on a Zeiss Duoscan with constant acquisition conditions to enable direct comparisons of direct mean fluorescence intensity (MFI) values. MFI values were measured with ImageJ, background subtracted, and then data were rescaled to a 0-100 scale of arbitrary units (A.U.). An arbitrary threshold (MFI >4) in the GFP channel was set for determining whether a cell was positive for ERdj3-sfGFP expression. (A) Images of representative cells in a field exhibiting no, high, and moderate ERdj3-sfGFP expression, respectively. In the top panel, differences in ERdj3-sfGFP expression are apparent. In the lower panel, the contribution of ERdj3-sfGFP to the total level of ERdj3 in cells is shown by anti-ERdj3 staining, which recognizes both endogenous and the sfGFP tagged ERdj3. Quantitation of the examples is shown in the table below. (B) Plot of MFI values of anti-ERdj3 intensities in cells expressing or not expressing ERdj3-sfGFP or not transfected. Mean values ± S.E.M. were: ERdj3-sfGFP 13.4 ± 1.1, no detectable GFP 6.6 ± 0.8, and untransfected 8.1 ± 0.4. FRAP data in Figures 4, 5, and 7 were generally collected in the range of the moderate ERdj-sfGFP expressing cells.
Guo and Snapp Supplemental Figure 1

A

ER-RFP $D$ ($\mu m^2/s$)

8.7±0.6 7.1±0.8

ER-RFP + ERdj3-sfGFP alone

B

ERdj3-sfGFP $D$ ($\mu m^2/s$)

0.20±0.01 0.12±0.01 0.11±0.01

C

Gain Setting

wt H53Q

0.20±0.01 0.19±0.01

with BiP-mCherry

p<0.0001

p=0.002

Guo and Snapp Supplemental Figure 1
Guo and Snapp Supplemental Figure 3

<table>
<thead>
<tr>
<th>MFI (A.U.)</th>
<th>ERdj3-sfGFP</th>
<th>α-ERdj3</th>
</tr>
</thead>
<tbody>
<tr>
<td>no detectable GFP</td>
<td>0.1</td>
<td>7.6</td>
</tr>
<tr>
<td>untransfected</td>
<td>14.7</td>
<td>18.2</td>
</tr>
<tr>
<td>ERdj3-sfGFP +</td>
<td>7.5</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Expression levels:
- no expression
- high expression
- moderate expression