SDF2-like protein 1 (SDF2L1) regulates the endoplasmic reticulum localization and chaperone activity of ERdj3 protein

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Running title: SDF2L1 enhances ERdj3 chaperone activity

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ABSTRACT
Molecular chaperones facilitate protein folding by associating with nascent polypeptides, thereby preventing protein misfolding and aggregation. Endoplasmic reticulum (ER) chaperone BiP, the sole HSP70 chaperone in the ER, is regulated by HSP40 chaperones, including ER-resident protein ERdj3 (DNAJB11). ERdj3 lacks an ER retrieval signal, is secreted under ER stress conditions, and functions as a chaperone in the extracellular space, but how its secretion is regulated remains unclear. We recently showed that ERdj3 forms a complex with ER-resident stromal cell–derived factor 2 (SDF2) and SDF2L1 (SDF2-like protein 1) and thereby prevents protein aggregation during the BiP chaperone cycle. However, the contribution of the ERdj3–SDF2L1 complex to protein quality control is poorly understood. Here, we analyzed the intracellular localization and chaperone activity of ERdj3 in complex with SDF2L1. We found that ERdj3 was retained in the ER by associating with SDF2/SDF2L1. In vitro analyses revealed that the ERdj3 dimer incorporated two SDF2L1 molecules; otherwise, ERdj3 alone formed a homotetramer. The ERdj3–SDF2L1 complex suppressed ER protein aggregation, and
this suppression did not require substrate transfer to BiP. The ERdj3–SDF2L1 complex inhibited aggregation of denatured glutathione S-transferase (GST) in vitro and maintained GST in a soluble oligomeric state. Both in cellulo and in vitro, the chaperone activities of the ERdj3–SDF2L1 complex were higher than those of ERdj3 alone. These findings suggest that, under normal conditions, ERdj3 functions as an ER chaperone in complex with SDF2/SDF2L1, but is secreted into the extracellular space when it cannot form this complex.

In eukaryotes, approximately one-third of cellular proteins are co-translationally transported into the lumen of the ER, where nascent polypeptides accept post-translational modifications that enable them to fold and assemble properly (1-3). Molecular chaperones assist in the folding of polypeptides to prevent misfolding and aggregation (4,5). Several proteins, including immunoglobulin κ light chain (κ LC) (6), contain intrinsically unstructured regions that are prone to aggregate unless they can interact with suitable binding partners (4,6). Accumulation and aggregation of misfolded proteins in the ER disrupts cellular homeostasis and activates the unfolded protein response (UPR), which upregulates molecules involved in protein quality control to restore ER homeostasis (2,7,8). If misfolded proteins continue to accumulate in the ER, the UPR eventually triggers apoptosis (9,10).

ERdj3/DNAJB11 is an ER-resident homologue of the E. coli DnaJ protein. It was originally identified as the HSP40 co-chaperone HEDJ involved in Shiga toxin trafficking (11,12). ERdj3 is an ER-luminal glycoprotein that forms a homotetramer and contains a conserved J domain that is essential for its interaction with BiP, a central player in the ER HSP70 cycle (4,13). Specifically, ERdj3 promotes the ATPase activity and subsequent conformational change of BiP (14,15). In addition, ERdj3 directly binds to unfolded proteins and transfers them to BiP, thereby preventing its substrates from aggregating (16-18). ERdj3 is upregulated by the UPR (13,14), and is secreted along with misfolded proteins when the UPR is active (19). Secreted ERdj3 associates with aberrant conformers and prevents them from aggregating in the extracellular space (14,19). Thus, ERdj3 is involved not only in ER homeostasis, but also in extracellular proteostasis. However, the mechanisms underlying regulation of ERdj3 localization remain to be elucidated.

SDF2 was originally identified in a mouse stromal cell line (20), and SDF2L1 was later cloned as a homologue of SDF2 (21). SDF2 and SDF2L1 have three MIR domains, which are also found in protein O-mannosyltransferase [PMT], inositol 1,4,5-trisphosphate receptor [IP3R], and ryanodine receptor [RyR] (22). These domains are predicted to form typical β-trefoil structures, which is important for protein stability (23). The β-trefoil structure is conserved in the SDF2 family (24), but the functions of the MIR domains remain elusive. SDF2L1 mRNA is significantly upregulated by the UPR, whereas SDF2 is constitutively expressed (21,25,26). SDF2L1 interacts with an ER chaperone complex containing BiP, ERdj3, and folding enzymes, suggesting a role in ER quality control of nascent
proteins (25,27). SDF2 and SDF2L1 contain ER retrieval signals (28,29) and are stabilized by interaction with ERdj3 (25). However, it is unclear how the ERdj3–SDF2L1 complex contributes to ER homeostasis, and little is known about the biological function of SDF2 and SDF2L1.

In this study, we analyzed the chaperone activity of the ERdj3–SDF2L1 complex both in cellulo and in vitro, and found that SDF2L1 promotes the chaperone activity of ERdj3, which inhibits protein aggregation. We also showed that SDF2 and SDF2L1 are required for ER localization of ERdj3 under physiological conditions. Our results indicate that SDF2 and SDF2L1 regulate the intracellular localization of ERdj3, and that ERdj3 retained in the ER acquires elevated chaperone activity by forming a complex with SDF2 or SDF2L1.

Results

The ERdj3–SDF2L1 complex inhibits aggregation of misfolded proteins

Immunoglobulin κ light chain (κ LC) is a misfolding-prone protein with a partially folded structure (6,30). In the absence of Ig heavy chain, κ LC is retained in the ER in association with BiP (6), which is subsequently degraded by ERAD (30). However, when proteasome function is abrogated, κ LC is retained in the ER for a prolonged period (31). We predicted that κ LC retained in the ER would form aggregates. To test this idea, we treated HEK293 cells expressing κ LC with MG132, a proteasome inhibitor, and separated 1% NP-40–soluble (S) and –insoluble (P) fractions. In control cells, about half of κ LC was recovered in the pellet fraction, but the proportion of protein in the pellet was greatly decreased by overexpression of BiP (Fig. 1A, compare lanes 3 and 4 with 1 and 2, quantified in B). Thus, chaperone activity in the ER can be assessed based on the reduction in κ LC aggregation. Using this assay, we compared the impact of the ERdj3–SDF2L1 complex with that of ERdj3 or SDF2L1 expressed alone in HEK293 cells. ERdj3 or SDF2L1 alone did not affect the P/S ratio of κ LC, whereas the ERdj3–SDF2L1 complex decreased the amount of κ LC in the pellet fraction to a similar extent as BiP (Fig. 1A, lanes 5–10, quantified in B). These results suggest that the ERdj3–SDF2L1 complex has strong chaperone activity, comparable to that of BiP, in cellulo. Furthermore, even in the absence of MG132, knock-down of both endogenous SDF2 and SDF2L1, but not that of ERdj3, increased the amount of κ LC in the pellet fraction (Fig. S1), supporting the importance of the ERdj3–SDF2L1 complex.

We next examined the effect of the ERdj3–SDF2L1 complex on terminally misfolded NHK-QQQ (non-glycosylated α1-antitrypsin, null Hong Kong variant) (32,33). NHK-QQQ associates with BiP (34) and is subsequently degraded by ERAD (33). In control cells, approximately half of NHK-QQQ accumulated in the 1% NP-40–insoluble fraction in the absence of proteasome inhibitor (25), and this proportion was significantly decreased by co-expression of BiP (Fig. 1C, lanes 1–4, quantified in D). Co-expression of the ERdj3–SDF2L1 complex, but not ERdj3 or SDF2L1 alone, decreased the amount of NHK-QQQ in the pellet fraction (Fig.
1C, lanes 5–10, quantified in D). Thus, BiP, ERdj3, and SDF2L1 affected NHK-QQQ aggregation similarly to κ LC aggregation. Notably, ERdj3 alone did not inhibit the aggregation of κ LC and NHK-QQQ in a cell-based assay. Considering the well-established chaperone activity of ERdj3, which binds and transfers unfolded or misfolding-prone proteins to BiP in cells (14,17,35), our results strongly suggest that ERdj3 requires an additional factor, SDF2L1, for its chaperone activity in the ER.

**SDF2L1 is required for ER localization of ERdj3**

ERdj3 is secreted into the extracellular space when its expression is upregulated due to ER stress or overexpression (19). ERdj3 lacks an ER retrieval signal (Fig. 2A, left), whereas SDF2L1 is retained in the ER via a C-terminal HDEL motif (28) (Fig. 2A, right). Hence, we analyzed the subcellular localization of the ERdj3–SDF2L1 complex by immunocytochemistry. To distinguish the transfected proteins from their endogenous counterparts, we fused the myc-tag and HA-tag to ERdj3 and SDF2L1, respectively, and visualized the transfected proteins using anti-tag antibodies. Staining for overexpressed ERdj3-myc was localized in the extracellular space (Fig. 2B, upper). The myc-tag signal did not co-localize with the ER marker PDI, and was detectable without cell permeabilization (Fig. 2C). However, ERdj3-myc was retained in the ER by co-expression of SDF2L1-HA (Fig. 2B lower), indicating that secretion of ERdj3 was inhibited by formation of a complex with SDF2L1 in the ER.

We next performed cycloheximide chase experiments to monitor the secretion of ERdj3. ERdj3-myc in cell lysate decreased, with a reciprocal increase in the medium, during the chase periods (Fig. 3A, lanes 1–3, 7, and 8, quantified in B, open circle). Co-expression of SDF2L1 increased the amount of ERdj3 remaining in the cell lysate, and conversely decreased the amount of ERdj3 secreted into the medium (Fig. 3A, lanes 4–6, 9, and 10, quantified in B, closed circles). These results prompted us to analyze the requirement of SDF2L1 for the ER localization of endogenous ERdj3. Knockdown of SDF2L1 using small interfering RNA (siRNA) increased the secretion of ERdj3, although the difference was not statistically significant (Fig. 3C, compare lane 7 with 5, quantified in D). We speculated that the small effect of SDF2L1 knockdown was caused by the lower expression of SDF2L1 in HEK293 cells under normal growth conditions (GeneCards: https://www.genecards.org/cgi-bin/carddisp.pl?gene=SDF2L1, and (25)). Therefore, we depleted SDF2, a homologue of SDF2L1, that also interacts with ERdj3 in cells (25). Knockdown of SDF2 increased secretion of ERdj3 into the medium to a greater extent than knockdown of SDF2L1 (Fig. 3C, compare lane 6 with 7, quantified in D). Depletion of both SDF2 and SDF2L1 further potentiated the secretion of ERdj3 (Fig. 3C, compare lane 8 with 5, quantified in D). Consistent with this, the level of ERdj3 in the cell lysate was significantly reduced when both SDF2 and SDF2L1 were knocked down (Fig. 3C, compare lane 4 with 1, IB: ERdj3). These results indicate that SDF2L1 and SDF2 are
redundantly required for the ER localization of ERdj3.

**ERdj3 chaperone activity in the ER requires an ER retrieval signal and complex formation with SDF2L1**

We next investigated whether the HDEL ER retrieval signal of SDF2L1 was required for ER localization of ERdj3. SDF2L1 lacking the C-terminal HDEL sequence (SDF2L1-ΔC) was secreted in the medium (Fig. S2). Consistent with this, co-expression of SDF2L1-ΔC did not inhibit secretion of ERdj3 (Fig. 4A, compare lanes 8 and 9, IB: ERdj3), and vice versa (Fig. S2). ERdj3 bound SDF2L1-ΔC similarly to wild-type SDF2L1 (Fig. S3). We then compared the chaperone activities of ERdj3–SDF2L1-ΔC and wild-type ERdj3–SDF2L1 complexes using a cell-based assay. Co-expression of ERdj3 and SDF2L1-ΔC did not increase the solubility of κ LC (Fig. 4A, IB: κ LC, quantified in B), revealing that the ERdj3–SDF2L1-ΔC complex lost the ability to inhibit aggregation of misfolding-prone proteins in the ER. These results suggest two possibilities for the chaperone activity of the ERdj3–SDF2L1 complex in cells: either ERdj3 itself has the chaperone activity, and SDF2L1 is required for the ER retention of ERdj3, or alternatively, ERdj3 and SDF2L1 cooperatively create the chaperone activity by forming a complex.

To discriminate between these two mechanisms, we introduced a KDEL ER retrieval signal at the C-terminus of ERdj3 (ERdj3-KDEL) (19). Unlike wild-type ERdj3, ERdj3-KDEL inhibited aggregation of κ LC (Fig. 4C, lanes 1–4, quantified in D), indicating that ER-retained ERdj3 has chaperone activity toward aggregation-prone ER proteins. Notably, κ LC in the pellet fraction was further suppressed by co-expression of SDF2L1-ΔC (Fig. 4C, lanes 3–6, quantified in D), demonstrating that SDF2L1 promotes the chaperone activity of ERdj3 by forming a complex. Because endogenous ERdj3 was secreted when SDF2L1-ΔC was co-expressed, we could only estimate the effect of transfected ERdj3-KDEL–SDF2L1-ΔC complex. As expected, ERdj3-KDEL inhibited secretion of SDF2L1-ΔC (Fig. S2). Collectively, these results suggest that SDF2L1 promotes the chaperone activity of ERdj3, not only by retaining ERdj3 in the ER, but also by increasing ERdj3 activity through complex formation.

**Chaperone activities of ERdj3 and the ERdj3–SDF2L1 complex are independent of BiP in cells**

DnaJ proteins transfer unfolded substrates to HSP70 proteins (14,17,18). Because the ERdj3–SDF2L1 complex inhibited the aggregation of misfolding-prone proteins to a similar extent as BiP (Fig. 1), we investigated whether substrate transfer to BiP was required for the chaperone activity of ERdj3 and the ERdj3–SDF2L1 complex. The ERdj3 H53Q mutant, which cannot interact with BiP, lacks the ability to transfer substrates to BiP (13). ERdj3 H53Q-KDEL decreased the P/S ratio of κ LC to a similar extent as wild-type ERdj3-KDEL (Fig. 4C, compare lanes 7 and 8 with 3 and 4, quantified in D), consistent with previous reports that the J-domain mutation does not affect substrate binding (13,18). Importantly, co-expression of SDF2L1-
ΔC further decreased the amount of κ LC in the pellet fraction (Fig. 4C, compare lanes 9 and 10 with 7 and 8, quantified in D). These results suggest that the cooperative effect of SDF2L1, which increases ERdj3 chaperone activity, is independent of BiP.

**ERdj3 and SDF2L1 form a stoichiometric complex**

To clarify the mechanistic details underlying the cooperativity of ERdj3 and SDF2L1, we purified recombinant proteins expressed in *E. coli* for *in vitro* analysis. To estimate the stoichiometry of the ERdj3–SDF2L1 complex, we first examined the direct binding of ERdj3 and SDF2L1. In these experiments, ERdj3 and SDF2L1 were mixed at various molar ratios, incubated for 30 min, and separated by native PAGE (Fig. 5A), in which ERdj3, SDF2L1, and the ERdj3–SDF2L1 complex had different electrophoretic mobilities. ERdj3 forms a homodimer in cells and a homotetramer in the extracellular space (16,17,36). The ERdj3–SDF2L1 complex migrated faster than ERdj3 in native PAGE (Fig. 5A, compare open and closed triangles), suggesting that the ERdj3 oligomer has a higher molecular weight than the ERdj3–SDF2L1 complex. We confirmed that both ERdj3 and SDF2L1 were present in the ERdj3–SDF2L1 complex by western blot analysis (Fig. S4A).

We next mixed ERdj3 and SDF2L1 at different molar ratios, and after incubation for 30 min at room temperature, the mixture was fractionated by gel filtration (Fig. 5B). Fractions containing recombinant proteins were separated by SDS-PAGE and stained with CBB (Fig. 5C). The ERdj3 oligomer formed a peak around fraction 11, whereas the peak fraction of the ERdj3–SDF2L1 complex was 13 (Fig. 5B). These results further support the notion that the ERdj3 oligomer has a higher molecular weight than the ERdj3–SDF2L1 complex. Quantification of each protein stained by CBB revealed that the molar ratio of ERdj3 to SDF2L1 in the ERdj3–SDF2L1 complex was nearly 1:1 (Fig. 5C and S4B).

The calculated molecular weights of His<sub>6</sub>-ERdj3 and His<sub>6</sub>-SDF2L1 are 40230 and 23539, respectively. To measure the hydrodynamic radius of the ERdj3 oligomer and the ERdj3–SDF2L1 complex, we fractionated the recombinant proteins by glycerol gradient ultracentrifugation. The sedimentation coefficients of the ERdj3 oligomer and the ERdj3–SDF2L1 complex were calculated as 7.0 S<sub>20,w</sub> and 6.1 S<sub>20,w</sub>, respectively (Fig. 5D). Comparison with the sedimentation coefficients of standard proteins suggested that purified ERdj3 is a tetramer, and that it dissociates upon binding to SDF2L1. Thus, the ERdj3–SDF2L1 complex most probably consists of an ERdj3 homodimer and two SDF2L1 molecules.

To confirm the number of subunits in the ERdj3 oligomer and ERdj3–SDF2L1 complex, we purified cysteine-free SGFP2-tagged ERdj3 (cfSGFP2-ERdj3) (37) and analyzed single-molecule brightness using photon counting multiple histograms (PCMH) (38). Native PAGE revealed that cfSGFP2-ERdj3 formed a complex with SDF2L1 similar to that of ERdj3 (Fig. S4C). PCMH analysis revealed that the molecular brightness of cfSGFP2-ERdj3 was ~four times higher than that of monomeric cfSGFP2,
indicating that purified cfSGFP2-ERdj3 forms a tetramer (Fig. 5E). Importantly, after the addition of SDF2L1, the molecular brightness decreased to nearly half that of cfSGFP2-ERdj3 alone, indicating that the cfSGFP2-ERdj3–SDF2L1 complex must contain two cfSGFP2-ERdj3 molecules (Fig. 5E). Taken together, we conclude that in the presence of SDF2L1 the ERdj3 tetramer is converted to complex containing two subunits of ERdj3 and two subunits of SDF2L1.

We next sought to determine the oligomeric state of ERdj3 in complex with SDF2L1 in cells. In HEK293 cells expressing both SDF2L1-FLAG and SDF2L1-HA, SDF2L1-FLAG did not co-immunoprecipitate with SDF2L1-HA (Fig. S4D, lane 5, IP: HA), and vice versa (Fig. S4D, lane 8, IP: FLAG), indicating that SDF2L1 does not form a homodimer. However, in the presence of ERdj3, SDF2L1-HA and SDF2L1-FLAG co-immunoprecipitated with each other (Fig. S4D, lane 6, IP: HA, and lane 9, IP: FLAG). ERdj3-myc was also included in the immunoprecipitates (Fig. S4D, lanes 6 and 9, IB: myc). Given the stoichiometry that we had determined in vitro previously (1:1 ERdj3:SDF2L1), we concluded that, in the ER, each ERdj3 homodimer is associated with two SDF2L1 molecules.

**SDF2L1 increases the chaperone activity of ERdj3 in vitro**

Heat-denatured glutathione S-transferase (GST) forms amorphous aggregates, which can be monitored based on turbidity, and addition of chaperone proteins inhibits the GST aggregation (39). To analyze the chaperone activity of the ERdj3–SDF2L1 complex in vitro, we mixed ERdj3:SDF2L1 at a molar ratio of 2:1 and monitored the turbidity of GST after incubation at 43°C. Addition of the ERdj3–SDF2L1 complex markedly inhibited the increase in turbidity (Fig. 6A), whereas addition of SDF2L1 did not ameliorate aggregation (Fig. 6A). These results suggest that the ERdj3–SDF2L1 complex has a chaperone activity that inhibits the aggregation of heat-denatured GST, whereas SDF2L1 alone does not. Unexpectedly, however, addition of ERdj3 significantly increased the turbidity (Fig. 6B). We postulated that this increase was caused by the heat-induced aggregation of ERdj3, which easily formed aggregates during the purification process. Hence, after incubation at 43°C for 24 h, we separated the reaction mixture into soluble and aggregated fractions by centrifugation at 14,000 × g for 5 min, and then analyzed the fractions by SDS-PAGE. Almost all ERdj3 was recovered in the aggregated fraction (Fig. 6C, lane 3), whereas the ERdj3–SDF2L1 complex was present in the soluble fraction (Fig. 6C, lane 10). Thus, recombinant ERdj3 is heat-sensitive, and forms aggregates during incubation at 43°C. Importantly, the ERdj3–SDF2L1 complex decreased aggregation of GST (Fig. 6C, lane 5, IB: GST, quantified in D), consistent with the turbidity change we had observed. Interestingly, GST aggregation was not promoted even in the presence of aggregated ERdj3 (Fig. 6C, lane 3, quantified in D). Next, we estimated the stability of ERdj3 by thermal shift assay. ERdj3 had melt temperature peaks at 48 and 51.5°C, but no peak was detected for the ERdj3–SDF2L1 complex (Fig. S5). These results suggest that heat-labile ERdj3 is stabilized by the association of SDF2L1.
To avoid thermal denaturation of ERdj3, we used chemically denatured GST as a substrate. Specifically, guanidine-HCl–denatured GST was diluted in a reaction buffer containing recombinant proteins, and GST recovered in the soluble and aggregated fractions was quantified after separation by SDS-PAGE (Fig. 6E). All of the denatured GST formed aggregates after dilution in the buffer, but aggregation was significantly inhibited by addition of ERdj3 (Fig. 6E, compare lanes 5 and 6 with 1 and 2; IB: GST, quantified in F). Importantly, GST aggregation was further suppressed by dilution in a buffer containing the ERdj3–SDF2L1 complex (Fig. 6E, lanes 9 and 10, quantified in F), suggesting that the complex has stronger chaperone activity than ERdj3 alone.

The soluble fractions were separated by native PAGE and blotted using anti-GST antibody. In the presence of the ERdj3–SDF2L1 complex, denatured GST entered the native polyacrylamide gel with reduced mobility, suggesting formation of GST oligomers (Fig. 6G, lane 6). Native GST dimers were not detected (Fig. 6G, compare lane 6 with 1), indicating that denatured GST was maintained in a soluble oligomeric state in association with the ERdj3–SDF2L1 complex. On the other hand, in supernatant diluted in ERdj3-containing buffer, a fraction of denatured GST did not enter the native polyacrylamide gel, suggesting the existence of large oligomers (Fig. 6G, lane 6). These results demonstrate that both ERdj3 and the ERdj3–SDF2L1 complex maintain the denatured GST in soluble oligomeric states, and that the ERdj3–SDF2L1 complex further decreases the size of soluble oligomers of denatured GST.

Discussion

In the ER, newly synthesized proteins assume their correctly folded conformations with the assistance of molecular chaperones. ERdj3 is a canonical HSP40 co-chaperone that binds to misfolded proteins in the ER. Previously, we reported that SDF2 and SDF2L1 form a complex with ERdj3 and contribute to the BiP chaperone cycle (25). In the present study, we analyzed the chaperone activity of the ERdj3–SDF2L1 complex both in cellulo and in vitro, and demonstrated that this complex has higher chaperone activity than ERdj3 alone.

We first performed a cell-based chaperone activity assay using HEK293 cells and found that transient expression of the ERdj3–SDF2L1 complex, but not ERdj3 or SDF2L1 alone, inhibited aggregation of misfolding-prone proteins in the ER (Fig. 1). Further analysis revealed that SDF2L1 promoted the chaperone activity of ERdj3 by two mechanisms: first, by retaining ERdj3 in the ER, and second, by increasing the chaperone activity of ERdj3 itself. ERdj3, which lacks an ER retrieval signal, is secreted into the extracellular space when the UPR is activated; there, it serves to maintain extracellular proteostasis (19). Here, we showed that co-expression of SDF2L1 inhibited the secretion of ERdj3, whereas knockdown of endogenous SDF2L1 and SDF2 had the opposite effect (Figs. 2 and 3). Thus, under normal growth conditions in HEK293 cells, ERdj3 is retained in the ER in association with SDF2 and SDF2L1. Both ERdj3 and SDF2L1 are upregulated by the
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UPR, and the induction of SDF2L1 is extensive (13,21,26), whereas SDF2 is a constitutively expressed protein (25,26). Hence, it is possible to speculate that, under ER stress, ERdj3 is retained in the ER to accommodate the higher protein load in the organelle. SDF2 could replace SDF2L1 both in cells and in vitro (Fig. S6). Collectively, our findings suggest that cells may regulate the localization of ERdj3, to maintain homeostasis, by controlling the expression levels of ERdj3 versus SDF2 and SDF2L1. Of note, we detected a fraction of secreted ERdj3 localized on the cell surface (Fig. 2), consistent with its function in extracellular signaling pathways, as previously reported for protogenin (40).

Using recombinant proteins, we revealed that ERdj3 binds directly to SDF2L1 at a molar ratio of 1:1 (Fig. 5C and S4B). ERdj3 forms a homotetramer in medium (36), in agreement with the results of our study. However, in the presence of SDF2L1, ERdj3 forms a heterotetramer composed of two ERdj3 and two SDF2L1 subunits (Fig. 5 and S4). These results may explain the previous observation that ERdj3 forms a dimer in cells (16), in which ERdj3 is complexed with SDF2 and/or SDF2L1 (Fig. S7). It is also possible to speculate that SDF2L1 may prevent ERdj3 dimer-dimer association. Further analysis is required to clarify these points.

To compare the chaperone activity of ERdj3 alone with that of the ERdj3–SDF2L1 complex in cells, we created an ERdj3 construct that was retained in the ER (ERdj3-KDEL) and estimated its effect on protein aggregation. ER-retained ERdj3 inhibited aggregation in the ER, but the ERdj3–SDF2L1 complex prevented the aggregation more efficiently (Fig. 4C and D), indicating that ERdj3 acquires higher chaperone activity upon complex formation with SDF2L1. Of note, substrate transfer to BiP was not required for the chaperone activities of ERdj3 and the ERdj3–SDF2L1 complex in cellulo (Fig. 4). Consistent with this, we detected in vitro chaperone activity of the ERdj3–SDF2L1 complex using chemically denatured GST as a substrate (Fig. 6E–G). The ERdj3–SDF2L1 complex inhibited the aggregation of denatured GST more efficiently than ERdj3 alone, further confirming that the chaperone activity of ERdj3 increased upon complex formation. We also detected chaperone activity of the ERdj3–SDF2L1 complex toward heat-denatured GST (Fig. 6A). However, ERdj3 itself formed aggregates during incubation at 43°C (Fig. 6B), and SDF2L1 alone did not inhibit the aggregation of denatured GST. Thus, there are two possible mechanisms for the increased chaperone activity of the ERdj3–SDF2L1 complex in vitro: first, ERdj3 has chaperone activity, and the binding of SDF2L1 stabilizes the conformation of the heat-labile ERdj3; or second, ERdj3 acquires higher chaperone activity upon complex formation with SDF2L1. Indeed, the heat stability of ERdj3 increased following complex formation with SDF2L1, as determined by thermal shift assay (Fig. S5). However, we favor the latter possibility because the aggregation of chemically denatured GST was more strongly inhibited by the ERdj3–SDF2L1 complex. ERdj3 and the ERdj3–SDF2L1 complex maintained denatured GST in the soluble state (Fig. 6), and soluble GST oligomers were smaller in the presence of the
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complex than in the presence of ERdj3 alone (Fig. 6G), further supporting the idea that the ERdj3–SDF2L1 complex functions as a holdase. Based on our findings in this study, we propose a mechanism in which the localization and chaperone activity of ERdj3 in the ER are regulated by complex formation with SDF2L1 (Fig. S7). Identification of the substrate-binding sites of ERdj3 and the ERdj3–SDF2L1 complex will be required to further analyze the molecular mechanisms underlying their chaperone activities.

ERdj3 retains the glucocerebrosidase mutants linked to Gaucher’s disease (41) and an α1-antitrypsin Z mutant that causes liver inflammation (42,43) in the ER. ERdj3 mutants that lead to an autosomal-dominant polycystic kidney disease have also reported (44). Our demonstration of the importance of SDF2L1 for the localization and function of ERdj3 may help to improve our understanding of these diseases.

Experimental procedures

Cell culture and transfection

HEK293 and COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml). Plasmids were purified using the Maxi- or Midi-prep plasmid purification kit (Qiagen) and transfected into cells using polyethyleneimine (branched; Sigma-Aldrich) or Lipofectamine 2000 (Invitrogen).

Construction of plasmids

NHK-QQQ, SDF2L1-HA, wild-type and myc-tagged ERdj3, and ERdj3 H53Q mutant were constructed as described previously (25,33). The immunoglobulin κ light chains from NS1 cells were a generous gift from Dr. L. M. Hendershot (St. Jude Children’s Research Hospital, USA). cDNA was generated from total RNA of HEK293 cells by RT-PCR. Human SDF2L1 and BiP were cloned into pcDNA3.1(+) by PCR using gene-specific primers. The HDEL deletion mutant of SDF2L1 (SDF2L1-ΔC) was constructed by PCR using wild-type SDF2L1 as a template. To generate ERdj3-KDEL and ERdj3 H53Q-KDEL, the KDEL sequence was added to the C-terminus of ERdj3 by PCR. For expression of recombinant proteins in E. coli, human ERdj3 and SDF2L1 lacking the N-terminal signal sequences were amplified by PCR and subcloned into pCold TF or pCold II vector (TaKaRa). To create cfSGFP2-ERdj3, cysteine-free SGFP2 (37) was amplified by PCR and subcloned into the N-terminus of the ERdj3 in the pCold II vector.

siRNA

The sequences of Stealth siRNA (Invitrogen) used to knock down endogenous proteins were as follows: SDF2, 5′-UGGCUGUAGUACCUCUGUGUUGUU-3′; and SDF2L1, 5′-CCACAACCAGGGAGUAGUGCCUU-3′ (25). Medium-GC (M) siRNA (Invitrogen) was used as a negative control. Each siRNA was transfected into cells at a concentration of 10 nM using Lipofectamine RNAi-MAX (Invitrogen) ~48 h before cell harvest.

Western blot analysis and immunoprecipitation

Western blot analysis was performed as described previously (25). Briefly, HEK293 cells lysed in a
buffer containing 1% NP-40 were centrifuged at 13,000 × g for 20 min at 4°C. The supernatant and pellet were collected as soluble and insoluble fractions, respectively. An equal volume of 2 × Laemmli containing 100 mM DTT was added to the supernatant, and after centrifugation, the pellet fraction was dissolved in Laemmli containing 50 mM DTT. Medium was collected after centrifugation at 500 × g for 5 min, and the supernatant was used for further analyses. Samples were separated by 10% or 12.5% SDS-PAGE and transferred to a PVDF membrane (Millipore). Specific signals were detected using Pierce Western blotting substrate (Thermo Scientific) on a LAS-4000 digital imaging system (GE Healthcare). Signal intensity was quantified using the ImageQuant software (GE Healthcare).

For immunoprecipitation, specific antibodies were added to cell extracts (1% NP-40–soluble fraction) prepared as described above, and then immune complexes were precipitated with Protein A– or Protein G–Sepharose (GE Healthcare) and eluted in Laemmli buffer containing 50 mM DTT. Immune complexes were separated by 12.5% SDS-PAGE and blotted onto PVDF membranes. The Clean-Blot IP detection reagent (Thermo Fisher Scientific) was used as the secondary antibody.

**Trichloroacetic acid (TCA) precipitation**

Serum-free medium was centrifuged at 500 × g for 5 min to remove the cell debris, and supplemented with 100 μg/ml BSA. TCA was added to the supernatant at a final concentration of 10% and incubated on ice for 10 min. The pellet obtained by centrifugation at 10,000 × g for 15 min at 4°C was washed with ice-cold ethanol. After drying on a vacuum concentrator, the pellet was dissolved in Laemmli buffer containing 50 mM DTT.

**Immunocytochemistry**

COS-7 cells plated on coverslips were transfected with the indicated plasmids. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde and permeabilized using 0.1% Triton X-100 where indicated. Primary antibodies were as follows: mouse monoclonal anti-HA (HA-7) (Sigma-Aldrich), rabbit anti-c-myc (A14) (Santa Cruz Biotechnology), and mouse monoclonal anti-PDI (Enzo Life Sciences). Signals were visualized by Alexa Fluor 594–conjugated anti–mouse IgG (Thermo Fisher Scientific) and Alexa Fluor 488–conjugated anti–rabbit IgG (Thermo Fisher Scientific). Nuclei were counterstained with DAPI. Images were acquired on a TCS SP8 confocal microscope equipped with a HC PL APO 63 × (NA1.4) objective lens (Leica Microsystems).

**Expression and purification of recombinant proteins**

To purify SDF2L1, *E. coli* (Origami B) cells transformed with pCold-TF-SDF2L1 were cultured at 37°C until OD₆₀₀ reached 0.6. The expression of recombinant protein was induced by cold shock at 15°C, and the cells were incubated for an additional 20 h in the presence of 0.4 mM isopropyl thio-β-d-galactoside (IPTG). Cell lysates was loaded onto a HisTrap column (GE Healthcare) equilibrated with buffer A (10 mM HEPES/KOH [pH 7.4], 150 mM NaCl, and 2 mM
CaCl₂) and eluted with buffer B (buffer A containing 500 mM imidazole). His₀-TF-SDF2L1 was further purified by gel filtration chromatography on a HiLoad 16/60 Superdex 200 (GE Healthcare). Fractions containing His₀-TF-SDF2L1 were dialyzed and digested with thrombin (Novagen) at 20°C for 16 h, and SDF2L1 was separated on a HiLoad 16/60 Superdex 200. For recombinant His₀-SDF2L1, E. coli (Origami B) cells transformed with pCold II-SDF2L1 were purified as described above for pCold-TF-SDF2L1, except that buffer C (80 mM HEPES/NaOH [pH 7.4], 500 mM NaCl, and 2 mM CaCl₂) and buffer D (buffer C containing 500 mM imidazole) were used for loading and elution on a HisTrap column, respectively. Next, His₀-SDF2L1 was separated on a HiLoad 16/60 Superdex 200, and the purified fractions were used for in vitro experiments. His₀-ERdj3 and His₀-cfSGFP2-ERdj3 were purified as described above for His₀-SDF2L1, except that E. coli (Origami B) cells transformed with pCold II-ERdj3 or pCold II-cfSGFP2-ERdj3 were cultured at 37°C until OD₆₀₀ reached 0.5, and then incubated at 15°C for 18 h in the presence of 0.2 mM IPTG. To obtain recombinant GST, E. coli (BL21) cells transformed with pGEX-5X-1 (GE Healthcare) were cultured at 37°C until OD₆₀₀ reached 0.6. Expression of recombinant protein was induced by adding 0.15 mM IPTG, and the cells were further incubated at 37°C for 4 h. GST bound to Glutathione Sepharose 4B (GE Healthcare) was eluted in buffer E (75 mM Tris/HCl [pH 8.0], 150 mM NaCl, and 20 mM GSH) and dialyzed against buffer C. The amounts of recombinant proteins were calculated based on their A₃₈₀ extinction coefficients.

**In vitro chaperone activity assay**

Aggregation of heat-denatured GST was monitored by measuring turbidity as described previously (39). Briefly, GST (final concentration, 8.5 μM) was incubated at 43°C with 6 μM BSA (Sigma), 4 μM His₀-ERdj3, 2 μM His₀-SDF2L1, or both 4 μM His₀-ERdj3 and 2 μM His₀-SDF2L1 in 96-well plates (675096, Greiner Bio-One) sealed with sealing film (676070, Greiner Bio-One). Absorbance at 355 nm was measured using an ARVO X3 (PerkinElmer) plate reader at 25°C. After the plate was shaken, the sealing film was removed prior to measurement. Absorbance at 0 h was subtracted from each datum. The reaction mixture, incubated for 24 h, was separated into soluble and aggregated fractions by centrifugation at 14,000 × g for 5 min at room temperature. An equal volume of 2 × Laemmli containing 200 mM DTT was added to the supernatant, and after centrifugation, the pellet fraction was first dissolved in 2 × Laemmli containing 200 mM DTT, and then an equal volume of reaction buffer was added to adjust the salt concentration. After separation by 12.5% SDS-PAGE, recombinant proteins were stained with CBB. To specifically detect GST signals, 1/10th volume of the samples used for CBB staining were blotted onto PVDF membranes after separation by SDS-PAGE, and then detected by western blotting with anti-GST antibody.

Aggregation of chemically denatured GST was analyzed by SDS-PAGE and native PAGE as described previously for denatured β-galactosidase (45). Recombinant GST denatured
with 6 M Guanidine-HCl was diluted 1:125 in reaction buffer (25 mM HEPES/NaOH [pH 7.5], 50 mM KCl, and 5 mM MgCl₂) to a final concentration of 40 nM. Prior to dilution, 6 μM BSA, 4 μM His₆-ERdj3, 2 μM His₆-SDF2L1, or both 4 μM His₆-ERdj3 and 2 μM His₆-SDF2L1 were added to the reaction buffer. After incubation at 37°C for 2 h, samples were separated to soluble and aggregated fractions by centrifugation at 14,000 × g for 5 min at room temperature. After separation by 12.5% SDS-PAGE or 8% native PAGE, recombinant proteins were stained with CBB or transferred to a PVDF membrane (Millipore). Specific signals were detected by western blot analysis using anti-GST antibody.

**Gel filtration chromatography**

The volumes of recombinant proteins were adjusted to 5 ml with Buffer C (7 μM His₆-ERdj3, 7 μM His₆-SDF2L1, 7 μM His₆-ERdj3 and 7 μM His₆-SDF2L1, or 7 μM His₆-ERdj3 and 3.5 μM His₆-SDF2L1). After incubation at room temperature for 30 min, samples were separated into 2 ml fractions on a HiLoad 16/60 Superdex 200 with buffer C using the AKTAprime plus system (GE Healthcare). Fractions containing recombinant proteins were separated by 12.5% SDS-PAGE and stained with CBB. Signal intensity was quantified using ImageQuant software.

**Glycerol density gradient ultracentrifugation**

Recombinant proteins were applied to a linear 5–25% glycerol density gradient and centrifuged at 157,000 × g in a swing-bucket rotor (RPS55; Hitachi) for 16 h at 4°C. Fractions were collected from the top, and after separation by SDS-PAGE, recombinant proteins were stained with CBB. The HMQ Calibration Kit for native electrophoresis (GE Healthcare) was used as a source of sedimentation markers.

**Single-molecule brightness analysis**

Single molecular brightness of cfSGFP2-ERdj3 was determined by photon counting multiple histograms (PCMH) (38), which measures the time-dependency of the photon counting histogram (PCH) (46), essentially as described previously (47). PCH analyzes fluctuations in fluorescence signal amplitude from an identical photon counting data used for fluorescence correlation spectroscopy (FCS). The PCH model describes the probability p of detecting k photon counts from a single fluorescent particle in a volume V₀ as

\[
p^{(1)}(k; V₀, ε) = \frac{1}{V₀} \int \text{Poisson}[k, ε \overline{PSF}(r)] d\overline{r},
\]

(1)

where ε is the single molecule brightness, \(\overline{PSF}(r)\) the scaled point spread function (PSF) at position \(r\), and \(\text{Poisson}(k,x)\) the Poisson distribution for k objects with a mean x. Total PCH containing N particles is calculated as the Nth convolution of (1):

\[
p^{(N)}(k; V₀, ε) = \left( p^{(1)} \otimes p^{(1)} \otimes \cdots \otimes p^{(1)} \right)(k; V₀, ε).
\]

(2)

In the original PCH model, molecule brightness is expressed by a specific brightness, \(ε_{sec} = ε/T\) (counts per second per molecule), which is independent of the arbitrary bin time T. However,
the actual fluorescence fluctuation is also caused by various processes including molecular diffusion or photophysical processes, affecting the parameters of PCH. Zare et al. introduced a function $\Gamma(T)$ to express the time dependency of $\varepsilon_{sec}$:

$$\varepsilon_{sec}(T) = \varepsilon_{sec}(0) \cdot \Gamma(T) \quad (3)$$

where

$$\Gamma(T) = \frac{1}{T^2} \int_{t_1}^{t_1 + T} \int_{t_1}^{t_2 + T} g_x(t_2 - t_1)dt_2dt_1. \quad (4)$$

Here $g_x(t)$ is the autocorrelation function for a process $x$ and $\varepsilon_{sec}(0)$ is the true specific brightness (38). $\Gamma(T)$ can be approximated using the corresponding $\Gamma(T)$ for triplet-state formation and 3D diffusion (38):

$$\Gamma(T) \approx I_{trip}(T)I_{dif}(T). \quad (5)$$

Assuming the process being stationary, the double integral for of Equation (5) can be reduced to a single integral form (6) (48)

$$\Gamma(T) = \frac{2}{T^2} \int_0^T (T - t)g_{trip}(t)g_{dif}(t)dt. \quad (6)$$

The solutions of each autocorrelation function are

$$g_{trip}(t) = \frac{1 - F_{trip} + F_{trip}e^{-t/\tau_{trip}}}{1 - F_{trip}},$$

and

$$g_{dif}(t) = \frac{1}{(1 + t/\tau_{trip})^2(1 + t^2/\tau_{dif}^2)},$$

where $F_{trip}$, $\tau_{trip}$, $\tau_{dif}$ and $s$ are the fraction and the relaxation time in the triplet state, 3D diffusion time, and the ratio of the axial to the lateral radius of the confocal observation volume.

To determine the oligomeric structures of ERdj3, the fluorescence signal of cSDF2-ERdj3 excited by a 488-nm diode laser in NIKON A1R was collected with a single-photon counting avalanche photodiode of PicoHarp300 (PicoQuant) through a bandpass filter of 520/35 nm with a 60 × water-immersion objective (numeric aperture 1.27). The apparent number of fluorescence molecules in the confocal volume (~0.35 fl) of the sample (10 μl) was set to 1–2 to obtain higher amplitudes of fluctuations. The concentration of total ERdj3 was adjusted with the His-tagged non-fluorescent molecule. From the photon counting data sets, each PCH was generated with 1st order out-of-focus correction at a series of bin times, and then the obtained $\varepsilon_{sec}(T)$ plot (PCMH) against the bin time was fit with the PCMH model (6) to obtain the bin time independence, $\varepsilon_{sec}(0)$, using FFS Data Processor ver.2.3 (SSTC, Minsk, Belarus) and Origin2019b (OriginLab).

**Antibodies**

Antibodies were purchased from the indicated suppliers: mouse monoclonal anti-SDF2 (Sigma-Aldrich), rabbit polyclonal anti-SDF2L1 (Sigma-Aldrich), rabbit polyclonal anti-ERdj3 (Sigma-Aldrich), goat polyclonal anti–mouse κ light chain (Southern Biotech), mouse monoclonal anti-HA (HA-7) (Sigma-Aldrich), rabbit polyclonal anti-HA (Recenttec), mouse anti-FLAG (M2) (Sigma-Aldrich), rabbit anti-FLAG (Sigma-Aldrich), rabbit anti–c-myc (A14) (Santa Cruz Biotechnology), sheep anti–α1-antitrypsin (The Binding Site), rabbit anti-calnexin (Enzo Life Sciences), mouse monoclonal anti-BiP (BD Transduction Laboratories), rabbit polyclonal anti-GST (MBL), horseradish peroxidase–conjugated anti–rabbit IgG (BTI), horseradish peroxidase–conjugated anti–mouse IgG (Zymed)
Laboratories Inc.), horseradish peroxidase–conjugated anti–sheep IgG (CAPPEL, Research Reagent), horseradish peroxidase–conjugated anti–goat IgG (Jackson ImmunoResearch).

**Statistical analysis**

All results are means ± SEM from at least three independent experiments. Statistical analysis was performed using the two-tailed Student’s *t*-test. Differences with *P* < 0.05 were considered statistically significant. *P* < 0.05; **P* < 0.01; ***P* < 0.001; and n.s., not significant.
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Author contributions: K.H., I.W. and N.H. designed, performed, and analyzed the experiments and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.
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**Figure 1.** Co-expression of ERdj3 and SDF2L1 inhibits aggregation of misfolded proteins. HEK293 cells were co-transfected with Ig κ LC and BiP, ERdj3, and/or SDF2L1, and then treated with MG132 for 24 h. Cells were lysed in buffer containing 1% NP-40 17 h after the addition of MG132, and NP-40–soluble (S) and –insoluble (P) fractions were separated by SDS-PAGE. The indicated proteins were detected by western blot analysis (A; quantified in B). Cells were transfected with NHK-QQQ and analyzed as in (A) without the addition of MG132 (C; quantified in D). Cells were harvested ~24 h after transfection. Data are means ± SEM of at least three independent experiments. pcDNA3.1(+) with no insert was used as a mock transfection control. Endogenous BiP (lanes 1, 5, 7, and 9), ERdj3 (lanes 1, 3, and 7), and SDF2L1 (lanes 1, 3, and 5) were also detected. **P < 0.01; *P < 0.05; n.s., not significant (two-tailed Student’s t-test).
Figure 2. SDF2L1 co-localizes with ERdj3 in the ER. (A) Domain organization of ERdj3 (left) and SDF2L1 (right). (B) COS-7 cells were transfected with ERdj3-myc (upper) or both ERdj3-myc and SDF2L1-HA (lower). Twenty-four hours after transfection, the cells were fixed, permeabilized, and subjected to immunostaining. ERdj3-myc was visualized with Alexa Fluor 488, and SDF2L1-HA or PDI (an ER marker) was visualized with Alexa Fluor 594. Nuclei were counterstained with DAPI. (C) COS-7 cells were transfected with ERdj3-myc and immunostained with anti-myc and anti-PDI antibodies without permeabilization. Myc-tag and PDI were visualized using Alexa Fluor 488– and 594– conjugated secondary antibodies, respectively. Nuclei were counterstained with DAPI. Scale bars, 10 μm.
Figure 3. SDF2L1 inhibits secretion of ERdj3 into the extracellular space. (A) HEK293 cells were transfected with SDF2L1-HA and/or ERdj3-myc, and medium containing cycloheximide (CHX) was added 24 h after transfection. Cell lysate and medium were collected after the indicated periods and separated by SDS-PAGE. Specific signals were detected by western blot analysis. (B) Quantification of ERdj3-myc in cell lysate (upper) and medium (lower) in (A). (C) HEK293 cells were treated with siRNA targeting SDF2, SDF2L1, or both SDF2 and SDF2L1, and serum-free medium was added 24 h after transfection. Cells were harvested 24 h after the medium change. Serum-free medium was concentrated by TCA precipitation. Samples were separated by SDS-PAGE, and specific signals were detected by
western blotting. Control: medium-GC negative control siRNA. Asterisk indicates a non-specific signal detected by anti-SDF2 antibody. (D) Signal intensities of ERdj3 in both the cell lysate and medium in (C) were adjusted to represent the same proportion of cells, and the medium/cell lysate ratio of ERdj3 was quantified. Data are means ± SEM of three independent experiments. **P < 0.01; *P < 0.05; n.s., not significant (two-tailed Student’s t-test).
Figure 4. SDF2L1 promotes ERdj3 chaperone activity in cells. (A) HEK293 cells were co-transfected with Ig κ LC, ERdj3, and either wild-type SDF2L1 or SDF2L1-ΔC (ΔC). Fresh medium containing MG132 was added 24 h after transfection, and the cells were incubated for 17 h. The cells were lysed in buffer containing 1% NP-40, and 1% NP-40–soluble (S) and –insoluble (P) fractions were separated by SDS-PAGE. Specific signals were detected by western blotting, and are quantified in (B). Endogenous ERdj3 appears in lane 1 of (A). SDF2L1 was immunoblotted on the same membrane used to detect κ LC. (C) HEK293 cells were co-transfected with Ig κ LC and the indicated plasmids, and fresh medium containing MG132 was added 24 h after transfection. Cells were lysed and analyzed as in (A) and quantified in (D). WK, wild-type ERdj3-KDEL; QK, ERdj3 H53Q-KDEL; ΔC, SDF2L1-ΔC. Data are means ± SEM from three independent experiments. **P < 0.01; *P < 0.05; ns, not significant (two-tailed Student’s t-test).
SDF2L1 enhances ERdj3 chaperone activity

A

His6-ERdj3

SDF2L1

2 4 1 2 4 8 µM

468

232

140

66

J3

J3-L1 complex

L1

B

C

Fr.

J3:L1=1:1

J3:L1=2:1

J3

L1

CBB

4.6 S

7.7 S

11.3 S

Top

4 6 8 10 12 14 16 18 20

Bottom

25 26 27 28 29

CBB

D

E

Apparent specific brightness (cpm)

0 2

Bin time (msec)

Corrected specific brightness (cpm)

0 5000 10000 15000 20000 25000 30000 35000 40000 45000 50000

0 2 4 6 8 10 12 14 16 18 20

cfSGFP2-ERdj3 cfSGFP2-ERdj3 cfSGFP2

His6-ERdj3 His6-ERdj3 His6-SDF2L1
Figure 5. ERdj3 and SDF2L1 bind directly to form a stoichiometric complex. (A) Recombinant SDF2L1 and His6-ERdj3 were mixed at the indicated concentrations and incubated at room temperature for 30 min. After separation by 8% native PAGE, recombinant proteins were stained with Coomassie Brilliant Blue (CBB). (B) Recombinant proteins [7 μM His6-ERdj3 (J3), 7 μM His6-SDF2L1 (L1), 7 μM His6-ERdj3 and 7 μM His6-SDF2L1 (J3:L1=1:1), or 7 μM His6-ERdj3 and 3.5 μM His6-SDF2L1 (J3:L1=2:1)] were fractionated by gel filtration chromatography, and the A280 profiles are shown. (C) Fractions containing recombinant proteins in (B) were separated by 12.5% SDS-PAGE and stained with CBB. (D) Glycerol density gradient sedimentation of fraction 11 of ERdj3, fraction 26 of SDF2L1, and fraction 13 of the ERdj3–SDF2L1 complex fractionated in (B) and (C). After separation by 12.5% SDS-PAGE, each protein was stained with CBB. Sedimentation markers used were BSA (4.6 S20,w), lactate dehydrogenase (7.7 S20,w) and catalase (11.3 S20,w) (49,50). (E) Photon counting multiple histogram assay of cfSGFP2-ERdj3 and the cfSGFP2-ERdj3–SDF2L1 complex. Recombinant cfSGFP2-ERdj3 protein (39 nM) was incubated with His6-ERdj3 (5.5 μM) alone or with His6-ERdj3 (5.5 μM) and His6-SDF2L1 (6.6 μM) for 30 min at room temperature and the fluorescence signal was recorded to obtain ~8000 events. Purified cfSGFP2 was used as a monomeric control. PCH was generated from the data sets at the indicated bin time and time dependency was analyzed as described under Experimental procedures. Each point represents the result of independent measurements (n=5).
Figure 6. The ERdj3–SDF2L1 complex inhibits GST aggregation. (A) and (B) GST (final concentration, 8.5 μM) was incubated with 50 μg of BSA, 4.5 μM His6-ERdj3 (J3), 2.25 μM His6-SDF2L1 (L1), or both 4.5 μM His6-ERdj3 and 2.25 μM His6-SDF2L1 (J3 + L1) at 43°C. Turbidity was monitored for the indicated time periods. Results are shown as means ± SEM from three independent experiments. (C)
and (D) Samples in (A) and (B) incubated for 24 h were fractionated into supernatant and aggregation fractions by centrifugation. After separation by SDS-PAGE, GST was detected using anti-GST antibody. BSA and recombinant proteins were stained with CBB. Aliquots (1/10th volume) of samples used for CBB staining were subjected to western blotting. Asterisks indicate non-specific signals. (D) Quantification of the aggregation/supernatant ratio of GST, as determined by western blotting in (C). Data are means ± SEM from three independent experiments. (E) Denatured recombinant GST was diluted 125-fold (final concentration, 40 nM) in reaction buffer containing 6 μM BSA, 4 μM His6-ERdj3 (J3), 2 μM His6-SDF2L1 (L1), or both 4 μM His6-ERdj3 and 2 μM His6-SDF2L1 (J3 + L1). After incubation at 37°C for 2 h, soluble (S) and aggregation (P) fractions were separated by SDS-PAGE. GST signals were detected by western blotting (IB: GST). BSA, ERdj3, and SDF2L1 were stained with CBB. The P/S ratio of GST is quantified in (F). Data are means ± SEM from five independent experiments. (G) Soluble fractions prepared in (E) were separated by 8% native PAGE and blotted onto a PVDF membrane. GST signals were detected by western blotting. Molecular weight standards for native PAGE are shown on the left. Native GST: non-denatured GST dimer. ***P < 0.001; **P < 0.01; and n.s., not significant (two-tailed Student’s t-test).
SDF2-like protein 1 (SDF2L1) regulates the endoplasmic reticulum localization and chaperone activity of ERdj3 protein

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