

Structural basis of Zn²⁺-mediated tethering that stabilizes ERp44-client complexes in protein quality control

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Abstract

ERp44 plays a critical role in protein quality control in the early secretory pathway by retrieving ER enzymes and immature secretory proteins from the Golgi to the ER. This function is regulated by Zn²⁺- and pH-dependent conformational changes in ERp44. However, the detailed mechanisms by which Zn²⁺ governs client binding in the Golgi and client release in the ER remain to be elucidated. Here, we report cryo-EM structures of complexes between Zn²⁺-bound ERp44 and two representative clients, ERAP1 and Ero1 α . These structures reveal that, beyond the mixed disulfide-mediated interactions, Zn²⁺ is coordinated by conserved histidine residues of ERp44 together with residues in client loops, forming two distinct types of Zn²⁺-mediated tethers that stabilize the complexes. Consistently, removal of Zn²⁺ promotes complex dissociation more effectively than reduction of the mixed disulfide bonds. In the physiological ER environment, millimolar concentrations of glutathione, together with picomolar levels of labile Zn²⁺ maintained by the ER-localized Zn²⁺ transporter ZIP7, promote complex dissociation by removing Zn²⁺ from the complex. The present findings demonstrate that Zn²⁺ plays dual roles in protein quality control by allosterically activating ERp44 and directly mediating client binding.

Introduction

A zinc ion (Zn²⁺) plays crucial roles in numerous biological processes¹. It acts as a catalytic center for numbers of enzymes or a structural cofactor that stabilizes protein structures^{2,3}. It also serves as a signal transducer in various cellular processes^{4,5}. Imbalance in Zn²⁺ homeostasis within the cells is highly related to various diseases⁶. In eukaryotic cells, Zn²⁺ levels are tightly controlled by two Zn²⁺ transporter families, ZnT (zinc transporter, SLC30) and ZIP (Zrt/Irt-like protein, SLC39)^{7,8}. The ZnT family members export Zn²⁺ from the cytosol into the organelles or the extracellular space, whereas members of the ZIP family import Zn²⁺ into the cytosol.

In the early secretory pathway (ESP), four ZnT family members (ZnT7, ZnT5, ZnT6, and ZnT4) and several ZIP family members are localized to the Golgi, maintaining sub-micromolar levels of labile Zn²⁺ in this organelle^{9,10}. In contrast, the labile Zn²⁺ concentration is maintained at picomolar levels in the endoplasmic reticulum (ER)¹¹, primarily through the action of ZIP7¹². Zn²⁺ transported into the Golgi participates in multiple processes, including its incorporation into Zn²⁺-dependent enzymes such as alkaline phosphatase (ALP)^{13–15}, regulation of ER-resident chaperone assembly^{16,17}, and promotion of cargo release from cargo receptors¹⁸.

ERp44, a member of the PDI family, functions as a second checkpoint for protein quality control by monitoring secretory proteins transported to the Golgi¹⁹. In the Golgi, ERp44 captures target clients, in most cases, via a mixed disulfide bond between its conserved Cys29 and a cysteine residue in the clients^{20–22}. This chaperone recognizes isolated subunits of immature oligomeric secretory proteins such as Immunoglobulin M (IgM) and adiponectin, as well as ER-resident enzymes such as ER oxidoreductin-1 α (Ero1 α), Peroxiredoxin 4 (Prx4), and ER aminopeptidase 1 (ERAP1). Lacking a C-

terminal ER-retention motif, they rely on ERp44 to maintain their localization within the ER^{23–27}. The ERp44-client complexes are retrogradely transported to the ER with the help of KDEL receptors (KDELRs).

Recently, we revealed that the large Zn²⁺ concentration gap between the ER and Golgi regulates the structure and function of ERp44^{10,28}. ERp44 consists of three thioredoxin (Trx)-like domains (**a**, **b**, and **b'** domains) and a long C-terminal tail (C-tail)²⁹. The **b'** domain of ERp44 contains a histidine cluster (His-cluster), which binds Zn²⁺ with sub-micromolar affinity (Fig. 1a, Supplementary Fig. 1a)²⁸. The crystal structure of Zn²⁺-bound ERp44 revealed that transient Zn²⁺ binding to the His-cluster induces conformational changes that open the C-tail, promoting tight association with its target clients (Supplementary Fig 1a). Subsequently, KDELRs retrogradely transport the ERp44-client complex to the ER, where the complex is most likely to dissociate. However, the molecular mechanisms underlying Zn²⁺-dependent client recognition by ERp44 in the Golgi and complex dissociation in the ER remain poorly understood.

Here, we report cryo-EM structures of Zn²⁺-bound ERp44 in complex with two representative clients, ERAP1 and Ero1 α , revealing Zn²⁺-mediated tethering at the complex interfaces, and highlighting the critical role of Zn²⁺ in regulating complex formation, stabilization and dissociation. These findings deepen our understanding of the physiological significance of Zn²⁺ in protein quality control within the ESP.

Results

Cryo-EM structures of the ERp44-ERAP1 complex

To elucidate how ERp44 recognizes target clients in a Zn²⁺-dependent manner, we performed cryo-EM analysis of the complex between ERp44 and one of its clients, ERAP1 (MW: 107 kDa). ERAP1 is an ER-localized aminopeptidase involved in peptide trimming for optimal major histocompatibility complex (MHC) class I loading³⁰. A previous report showed that Cys29 of ERp44 attacks the conserved cysteine pair Cys486–Cys496 of ERAP1, located in a long flexible loop, forming a mixed disulfide bond (Fig. 1b)²⁶. Consistent with our previous findings²⁸, addition of Zn²⁺ promoted formation of the ERp44-ERAP1 complex, which was used for cryo-EM analysis (Supplementary Fig. 1c, d). Ab initio reconstruction and 3D classification identified several distinct binding modes of the complex (Supplementary Fig. 2). Eventually, we determined cryo-EM structures of the ERp44-ERAP1 complex in two different binding modes (mode I and mode II) at a resolution of 3.75–4.58 Å (Fig. 1c–e). Particles that adopt mode I were further classified into two states (states 1 and 2).

In binding mode I, ERAP1 adopts an open conformation in which the catalytic site is exposed to the solvent (Fig. 1c, d, and Supplementary Fig 1b). Densities corresponding to the three Trx domains of ERp44 are clearly observed near the long flexible loop of ERAP1, although the overall density of ERp44 is

considerably weaker than that of ERAP1. In state 1 of mode I, the **b'** domain of ERp44 directly contacts ERAP1, and the helices $\alpha 9$ and $\alpha 11$ in the **b'** domain interact with the flexible loop that is partially resolved in the EM map (Fig. 1f). In state 2 of mode I, the **b'** domain of ERp44 is also located near the flexible loop of ERAP1, but at different angles from that observed in state 1 (Fig. 1g). Compared to the **b'** domain, the **a** and **b** domains are poorly resolved in the EM map (Fig. 1c, d), presumably due to their limited contacts with ERAP1, indicating high mobility of these domains within the complex.

In binding mode II, ERAP1 adopts a semi-closed conformation (Fig. 1e). The three Trx-like domains of ERp44 are positioned above the flexible loop of ERAP1 in the EM map. Notably, the orientation of ERp44 relative to ERAP1 in binding mode II is opposite to that observed in binding mode I, with the **b'** domain located on the left side of ERAP1 in this view (Fig. 1e). The $\alpha 9$ and $\alpha 11$ helices in the **b'** domain interact with the opposite side of the flexible loop (Fig. 1h), whereas the **a** domain makes very limited contact with ERAP1. In both binding modes I and II, the interfaces are poorly resolved due to limited resolution, precluding identification of Zn^{2+} -binding sites in ERp44 and the mixed disulfide bond between ERp44 and ERAP1.

Type I Zn^{2+} tether mediates the interaction between ERp44 and ERAP1

To further elucidate the detailed interface of the ERp44-ERAP1 complex, we collected an additional cryo-EM dataset using UltrAuFoil grids and determined its structure at a resolution of 3.12 Å (Fig. 2a, b, Supplementary Fig. 3a, b). In this dataset, only one Trx-like domain is clearly visible in the cryo-EM map (Fig. 2a). Among the three Trx-like domains, the **b'** domain fits well into the density map (Supplementary Fig. 4a), representing binding mode I. 3D variability analysis reveals the open-close conformational equilibrium of ERAP1 and a swinging motion of the **b'** domain with respect to ERAP1 (Fig. 2b, Supplementary Movie 1), suggesting that the ERAP1 loop involved in this interaction is flexible. Based on the updated cryo-EM map, a new model was built for residues Cys486-Thr488 and Ser506-Gly514 within the flexible loop of ERAP1 (Supplementary Fig. 4b), although these residues were missing in the previously published crystal structures³¹.

At the complex interface, His277 and His281 of ERp44 are positioned in close proximity to Cys486 and His509 of ERAP1 (Fig. 2c-e). Notably, residual density is observed at the center of this site, suggesting that a Zn^{2+} ion is coordinated by His277 and His281 of ERp44 and Cys486 and His509 of ERAP1, forming a Zn^{2+} -mediated tether (hereafter termed the 'type I' Zn^{2+} tether)(Fig. 2d). Our previous crystal structure analysis demonstrated that, in the absence of clients, His277 and His281 form a Zn^{2+} bridge at the dimer interface of the ERp44 homodimer²⁸ (Fig. 2f). Thus, the His277/His281 pair of ERp44 serves as a conserved Zn^{2+} -binding site that facilitates both homodimerization and heterodimerization. In contrast, the His cluster of ERp44 appears to lack Zn^{2+} (Supplementary Fig. 4c), suggesting that Zn^{2+} is transferred from the His cluster to the His277/His281 pair in this state. Adjacent to the type I Zn^{2+} tether, Trp510 of ERAP1 is accommodated in a hydrophobic pocket formed by Ile219, Phe234, Ile236, Phe238 and Phe275 of the **b'** domain of ERp44 (Fig. 2e). Hydrogen bonds between Asp516 of ERAP1 and Arg276 of ERp44, and between Asp513 of ERAP1 and the neighboring main chain of ERp44 further contribute to

their complex formation (Fig. 2e). Cys486, His509, and Trp510 of ERAP1 are highly conserved among ERAP1 orthologues (Fig. 2g). Thus, the ERp44-ERAP1 complex is stabilized by the type I Zn^{2+} tether formed by the conserved His/Cys residues of ERp44 and ERAP1, as well as by the hydrophobic pocket in the **b'** domain of ERp44.

Zinc removal, rather than disulfide bond reduction, is required for substantial dissociation of the ERp44-client complexes

To further explore the role of Zn^{2+} in stabilizing the ERp44-client complex, we next conducted dissociation analysis. Initially, we examined whether reduction of the mixed disulfide bond between ERp44 and its clients effectively triggers the complex dissociation, as previously suggested under Zn^{2+} -free conditions²⁰. To test this, the Zn^{2+} -tethered ERp44-ERAP1 complex was treated with dithiothreitol (DTT) and subjected to size-exclusion chromatography (SEC) (Fig. 3a). As expected, the mixed disulfide bond was substantially cleaved by DTT (Fig. 3b, lower). However, the majority of the proteins eluted as a single peak at the same elution volume as the covalent complex (Fig. 3b, upper), indicating that most of the complex was retained even after the cleavage of the mixed disulfide bond. Similar results were observed for another ERp44 client, Ero1 α (Fig. 3c). Upon DTT treatment, the reduced ERp44-Ero1 α complex eluted as a single peak that was slightly yet significantly narrower than that observed without DTT, suggesting that reduction of the mixed disulfide bond rather increases the homogeneity and stability of the binary complex. Collectively, reduction of the mixed disulfide bond alone was insufficient to release clients from ERp44.

Next, we investigated the effect of Zn^{2+} removal from the ERp44-client complex. To this end, the Zn^{2+} -tethered ERp44-ERAP1 complex was treated with a Zn^{2+} chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and subsequently analyzed by SEC (Fig. 3d). Upon Zn^{2+} removal from the complex, nearly half of the ERp44-ERAP1 complex dissociated (Fig. 3e). In the case of Ero1 α , TPEN treatment caused the proteins to elute predominantly as monomers, indicating that the ERp44-Ero1 α complex was fully disassembled upon Zn^{2+} removal (Fig. 3f). Thus, Zn^{2+} removal is key to the efficient dissociation of ERp44-client complexes.

His277/His281 of ERp44 are essential for binding ERAP1, but not for binding Ero1 α

Next, to assess the role of the His277/281 pair of ERp44 in the formation and stabilization of the complex with ERAP1, we conducted SEC analysis on a mixture of ERAP1 and the ERp44 His277/281Ala mutant (2HA mutant). As shown earlier, in the absence of Zn^{2+} , a significant portion of ERp44 can form the complex with ERAP1 (Supplementary Fig 1c), indicating that the Zn^{2+} tethering is not a prerequisite for initial complex formation. Given that the 2HA mutant retains the His cluster required for the Zn^{2+} -mediated C-tail opening²⁸, we inferred that the mutant would be capable of forming a complex with ERAP1 upon Zn^{2+} addition, but that the resultant complex would readily dissociate upon reduction of the mixed disulfide bond because the mutant no longer forms the type I Zn^{2+} tether with ERAP1.

Contrary to our expectation, SEC analysis showed that the ERp44 2HA mutant failed to form a complex with ERAP1 despite the addition of Zn^{2+} (Fig. 3g). This result indicates that the His277/281 pair of ERp44 serves a dual function: it not only participates in formation of the type I Zn^{2+} tether with ERAP1 but also plays an essential role in the initial recognition of ERAP1. To further investigate a general role of the His277/281 pair in complex formation, we tested whether the 2HA mutant can bind another client protein, Ero1 α , in the presence of Zn^{2+} (Fig. 3h). Intriguingly, the 2HA mutant retained the ability to form a Zn^{2+} -dependent complex with Ero1 α . The contrasting behavior of the 2HA mutant in complex formation with ERAP1 and Ero1 α suggests that whereas ERAP1 is initially recognized by ERp44 through the His277/281 pair, Ero1 α is bound by ERp44 via regions other than the His277/281 pair (see also the next section).

Cryo-EM structure of the ERp44-Ero1 α complex

To elucidate the mechanism by which ERp44 recognizes Ero1 α , we next performed cryo-EM analyses of the ERp44-Ero1 α complex. Our previous study reported that at least three types of mixed disulfide complexes can be formed between ERp44 and Ero1 α ²⁹. To prepare a homogenous ERp44-Ero1 α complex for structural study, we used a hyperactive Ero1 α mutant (Cys104Ala/Cys131Ala), in which Cys94 within the flexible long loop (Fig. 4a) is expected to uniformly form a mixed disulfide bond with Cys29 of ERp44³². The ERp44-Ero1 α complex was prepared in the presence of Zn^{2+} and subjected to grid preparation. Clear 2D class-averaged images were obtained from the grids of the PEG-treated complex (Supplementary Fig. 5). As was the case for the complex with ERAP1, *ab initio* reconstruction generated multiple binding modes of the complex (Supplementary Fig. 5). The initially determined cryo-EM maps of the complex exhibited severe orientation bias, resulting in low quality maps in which secondary structural elements were hard to resolve. Using computational orientation rebalance in CryoSPARC, followed by 3D refinement with Blush regularization³³, we successfully determined the cryo-EM structures of the ERp44-Ero1 α complex in three distinct binding modes (mode I, mode II, and the mode II dimer) at 3.65-4.2 Å resolutions (Fig. 4b,c and f, Supplementary Fig. 5, 6).

In binding mode I, ERp44 associates with Ero1 α through two contact sites (Fig. 4b). Although ERp44 **a** domain is located slightly distant from Ero1 α , a continuous density extends from Cys29 in the **a** domain of ERp44 to the main body of Ero1 α (Fig. 4d, left inset). The model of the Ero1 α loop built based on the density map indicates that Cys94 of Ero1 α forms a mixed disulfide bond with Cys29 of ERp44, consistent with previous biochemical experiments²⁹. A more extensive interface is formed between Ero1 α and the **b'** domain of ERp44 (Fig. 4d, right inset). The His cluster is located at this interface, suggesting that this cluster contributes directly to complex formation, although the side-chain densities were not clearly resolved due to the limited resolution.

In binding mode II, only ERp44 **a** domain contacts Ero1 α (Fig. 4c, e). The density map suggests that Cys29 of ERp44 forms a mixed disulfide bond, possibly with Cys94 of Ero1 α , although the density around these two cysteines is not clearly resolved (Fig. 4e left inset). There are few interactions between the **b'** domain of ERp44 and the loop of Ero1 α . Interestingly, significant extra densities were observed adjacent

to the **b'** domain, suggesting the existence of additional molecules bound to the ERp44-Ero1 α heterodimer (Supplementary Fig. 6). When particles assigned to binding mode II were re-extracted using a larger box size, a subset of the complexes was found to exist as a dimer of the Ero1 α -ERp44 heterodimer (Fig. 4f). The dimer interface is formed between the His cluster of ERp44 from one protomer and the His277/H281 pair of ERp44 from another protomer, likely via a Zn²⁺ coordination (Fig. 4f, right inset). However, no oligomers larger than a dimer were detected by SEC (Supplementary Fig. 5a), suggesting that the dimer of the Ero1 α -ERp44 heterodimer was only partly formed and readily dissociated.

Type II Zn²⁺ tether stabilizes the ERp44-Ero1 α complex

To further improve the resolution of the ERp44-Ero1 α complex in binding mode I, particles corresponding to this mode were merged from three datasets (Supplementary Fig 7). Based on the merged data, local classification focusing on Ero1 α and the **b'** domain of ERp44 was performed. Further local refinements significantly improved the cryo-EM map around the complex interface (Fig. 5a, b). At the interface between the **b'** domain and Ero1 α , two histidine residues (His299 and His328) from the His cluster are positioned near Glu142 and Glu177 of Ero1 α (Fig. 5c). The continuous density observed in this region indicates that Zn²⁺ is coordinated by His299 and His328 of ERp44 together with Glu142 and Glu177 of Ero1 α , forming another type of Zn²⁺-mediated tether (referred to as a 'type II' Zn²⁺ tether). These two glutamate residues are highly conserved among Ero1 α orthologues from rat to human (Fig. 5d). The third histidine residue of the His cluster, His332, participates in Zn²⁺ coordination in Zn²⁺-bound ERp44²⁸. Upon complex formation with Ero1 α , however, His332 is replaced by Glu142 of Ero1 α for Zn²⁺ coordination (Fig. 5e). In addition, the density map suggests that Glu132, Glu135 and Arg136 within the regulatory loop of Ero1 α interact with Leu280, His281, Pro286 and Gln283 of ERp44, contributing to complex formation (Fig. 5f). Thus, the interface between ERp44 and Ero1 α is primarily mediated by the type II Zn²⁺ tether.

To explore the importance of Glu142 and Glu177 of Ero1 α in complex formation, we performed SEC analysis using the E142A/E177A double mutant (2EA mutant) (Fig. 5g). This Ero1 α mutant retained Zn²⁺-dependent complex formation with ERp44 via a mixed disulfide bond between Cys29 (ERp44) and Cys94 (Ero1 α) (Fig. 5h, green curve). However, DTT-induced reduction of the mixed disulfide bond resulted in the dissociation of approximately half of the complex (Fig. 5h, red curve). This result contrasts sharply with those observed for wild-type Ero1 α , which exhibited only minimal dissociation upon reduction of the mixed disulfide (Fig. 3c). Thus, Glu142 and Glu177 of Ero1 α play considerable roles in complex formation with ERp44 via the type II Zn²⁺ tether. The residual complex species observed after DTT treatment may possibly be formed through other potential Zn²⁺-coordinating residues in Ero1 α .

Dissociation of the Zn²⁺-tethered ERp44-client complex in glutathione pool

As shown above, Zn^{2+} removal is required for the efficient dissociation of the ERp44-client complex (Fig. 3e, f). Next, we explored whether additional cellular factors may function as Zn^{2+} chelators that promotes Zn^{2+} removal from the complex in the ER environment. In this context, we focused on the cellular reducing compound, glutathione (GSH), as a potential Zn^{2+} chelator in the ER. This reducing tripeptide is abundant in the ER (>10 mM)^{34,35} and exhibits a weak chelating ability for Zn^{2+} through its thiol group^{36,37}. When pretreated with GSH, the ERp44-ERAP1 complex largely eluted in the same SEC fraction as the non-treated sample (Fig. 6b). However, when the GSH-pretreated ERp44-ERAP1 sample was subjected to SEC using a buffer containing 10 mM GSH, the sample eluted at a larger elution volume and exhibited a broader peak (Fig. 6b, red curve). A similar elution profile was observed for the ERp44-Ero1 α sample when using the GSH-containing buffer (Fig. 6c, red curve). Considering that Zn^{2+} removal by TPEN promotes complex dissociation (Fig. 3e, f), these broader elution profiles suggests that Zn^{2+} was depleted from the complex during the SEC in the presence of GSH. Together, these results suggest that the GSH pool can deplete Zn^{2+} from the ERp44-client complexes, thereby facilitating their dissociation.

Discussion

Our previous studies demonstrated that Zn^{2+} binding to the His cluster of ERp44 induces C-tail opening, thereby promoting its client binding (Fig. 6d right, step 1). The present cryo-EM structures of ERp44-client complexes further reveal that Zn^{2+} directly participates in client recognition between ERp44 and clients. In the ERp44-ERAP1 complex, the His277/His281 pair of ERp44, which engages in the Zn^{2+} bridge at the interface of the ERp44 homodimer (Fig 6d, step 2 and Supplementary Fig. 1a), is repurposed to form the type I Zn^{2+} tether with Cys486 and His509 of ERAP1 (Figs. 2d, 6d, step 3). The ERp44-Ero1 α complex is instead stabilized by the type II Zn^{2+} tether linking the His cluster of ERp44 to the two Glu residues (Glu142 and Glu177) of Ero1 α (Figs. 5c, and 6d, step 4). Consistently, our previous cell-based experiments demonstrated that mutations in either the His277/His281 pair or the His cluster in ERp44 impaired its client retrieval activity²⁸. Thus, the stable ERp44-client complexes are formed via at least two different types (type-I and type-II) of Zn^{2+} tether.

Intriguingly, AlphaFold 3 (AF3) prediction suggests that other clients can also be captured by ERp44 through Zn^{2+} -mediated tethering as well as mixed disulfide bonds (Supplementary Fig 8). In the predicted complex with the adiponectin trimer, Zn^{2+} is coordinated by the His277/His281 pair of ERp44 together with His46/His49 of adiponectin (Supplementary Fig 8a). In the predicted Prx4-ERp44 complex, Zn^{2+} is assumed to bind to the His277/His281 pair of ERp44 and Asp229 and Glu254 of Prx4 (Supplementary Fig 8b). The published structure of the ERp44-Prx4 complex prepared under Zn^{2+} -free condition suggests that a glutamate pair (Glu134 and Glu137) of Prx4 might participate in Zn^{2+} tethering with the His277/His281 pair of ERp44 under Zn^{2+} -available condition (Supplementary Fig 8c). In any case, the mode of recognition by ERp44 depends on the locations of Cys residues and Zn^{2+} -coordinating residues within the clients (Fig. 6e). These multiple binding modes may enable ERp44 to recognize a

wider range of clients, and possibly mediate rapid cycles of binding and release, for protein quality control.

Our mutational analyses provide further insights into how ERp44 recognizes the clients. In isolated state, ERAP1 Cys486 and Cys496 form a disulfide bond. The observation that reduced Cys486 of ERAP1 participates in the type I Zn^{2+} tether at the interface (Fig. 2d) suggests that the mixed disulfide formation between ERp44-Cys29 and ERAP1 Cys496²⁶ precedes the Zn^{2+} -mediated tethering. However, mutations at the key His277-His281 pair impaired mixed disulfide formation, preventing the complex formation. Collectively, binding mode II, which contains the mixed disulfide (Fig. 1e), likely represents the initial binding state, where the His277/His281 pair appears to play an auxiliary role in contact between the ERp44 **b'** domain and the ERAP1 main body (Fig. 1h). In the AF3 predicted model³⁸, the long flexible loop of ERAP1 adopts a U-shaped conformation (Fig. 1b). The Cys486-Cys496 disulfide bond is formed near the base of the U-shaped loop and does not appear to be readily accessible to the solvent. A docking model of the U-shaped loop on the mode II complex (Supplementary Fig. 9a) predicts potential steric hindrance between the ERp44 **b'** domain and the U-shaped loop, suggesting that the initial interaction mediated by the His277/His281 pair of ERp44 may induce conformational changes in the flexible loop of ERAP1, thereby exposing Cys486 and Cys496 to the solvent. Subsequently, Cys29 of ERp44 attacks Cys496 to form the mixed disulfide bond (Supplementary Fig 9b, steps 2 and 3). As a consequence, the flexible loop may adopt a more extended conformation and reposition the ERp44 domains, ultimately resulting in the formation of the type I Zn^{2+} tether at the interface between the ERp44 **b'** domain and the ERAP1 flexible loop (Supplementary Fig. 9b, step 4).

In the case of Ero1 α , both its crystal structure³² and AF3-predicted model suggests that the Cys94-Cys99 disulfide bond of Ero1 α is fully exposed to the solvent (Fig. 4a); therefore, the **a** domain of ERp44 can readily access this disulfide through electrostatic interaction with the target loop of Ero1 α ²⁹, leading to formation of the mixed disulfide bond between ERp44 Cys29 and Ero1 α Cys94 (Fig. 4e, Supplementary Fig 10 steps 1 and 2). In binding mode I, the complex is further stabilized by the type II Zn^{2+} tether involving the His-cluster of ERp44 (Fig. 5c, Supplementary Fig 10, step 4). In the binding mode II, the open conformation of ERp44, with Zn^{2+} bound to the His cluster, appears to be maintained through dynamic, transient dimerization (Fig 4f, right inset, Supplementary Fig 10, step 3). The formation of the mixed disulfide bond between ERp44 Cys29 and the catalytic Cys94 of Ero1 α explains how ERp44 binding inhibits PDI oxidation activity of Ero1 α , as observed previously³⁹.

After reaching the ER via retrograde transport, the complexes must dissociate to allow ERp44 to close its C-tail, release clients, and recycle back to the Golgi efficiently (Fig. 6e, left). The present finding that removal of Zn^{2+} from the complex effectively triggers complex dissociation is consistent with the presence of the Zn^{2+} -mediated tethering at the complex interface (Fig. 3). The complete dissociation of the ERp44-Ero1 α complex upon TPEN treatment (Fig. 1f) can be rationalized as follows; chelation of Zn^{2+} from the His cluster facilitates Ero1 α Cys99 to attack the mixed disulfide bond between ERp44 Cys29 and Ero1 α Cys94, thereby resolving the covalent complex. This reaction converts ERp44 into its

closed C-tail conformation with much lower affinity for the client (Fig. 6d, step 7, Supplementary Fig. 10 steps 5 and 6). In contrast, the ERp44-ERAP1 complex was not completely dissociated upon TPEN treatment (Fig. 3e). In this complex, the mixed disulfide bond between ERp44 Cys29 and ERAP1 Cys496 may be spatially distant from ERAP1 Cys486 in both binding modes I and II. Therefore, nucleophilic attack by ERAP1 Cys486 against the mixed disulfide proceed inefficiently and may require other reducing agents, such as GSH.

The labile Zn^{2+} concentration in the ER is maintained at the picomolar level¹¹, suggesting that Zn^{2+} is readily released from the ERp44-client complex once it is transported to the ER. However, Zn^{2+} is tightly coordinated in a tetrahedral geometry by the conserved histidine residues of ERp44 (Figs. 2d and 5c). Since Zn^{2+} binding affinity is likely higher under the neutral pH conditions of the ER than in the weakly acidic environment of the Golgi²⁸, dissociation of the complex cannot be explained by pH alone. This reasoning suggests that specific ER-resident factors function as Zn^{2+} chelators to promote Zn^{2+} removal from the complex.

In line with this, several studies have demonstrated that GSH participates in intracellular Zn^{2+} trafficking as well as disulfide bond reduction^{36,40,41}. Given that GSH and its oxidized form have a weak Zn^{2+} affinity³⁷, the presence of GSH at millimolar concentrations (~10 mM) is likely sufficient to dissociate the ERp44-ERAP1 and ERp44-Ero1 α complexes by extracting Zn^{2+} from their interfaces (Fig. 6b and c). Other low-molecular-weight ligands, such as ATP/ADP, also possess weak Zn^{2+} -binding capacity³⁷, and may similarly contribute to Zn^{2+} removal. Zn^{2+} extracted by GSH may subsequently be transferred to ER-resident Zn^{2+} -dependent enzymes or to the ER-localized Zn^{2+} transporter, ZIP7, which exports Zn^{2+} from the ER to the cytosol (Fig. 6d, step 8). ZIP7 itself may directly contribute to Zn^{2+} removal from the ERp44-client complexes (Fig. 6d, step 9). In support of this model, our recent study clearly demonstrated that inhibition of ZIP7 led to the accumulation of ERp44 bound to its clients, especially to Ero1 α ⁴². Taken together, these findings indicate that Zn^{2+} levels in the early secretory pathway are stringently regulated by the coordinated actions of Zn^{2+} transporters, chaperones and low-molecular-weight ligands, thereby ensuring proper protein quality control.

Methods

Plasmid and cell line constructions

The expression vectors for overexpression of ERp44 and Ero1 α were described previously²⁹. The Glu-to-Ala mutants of Ero1 α was constructed using PCR-based site-directed mutagenesis with appropriate sets of primers (Takara) (Supplementary Table 1). The cDNA of human ERAP1 was subcloned from a HeLa cell cDNA library and inserted into the pEF4 vector. A PCR fragment of the ERAP1 with a C-terminal FLAG tag was inserted into a pEBmulti-puro vector (Fujifilm-Wako) to generate a stable HEK293T cell line for ERAP1 overexpression. Human embryonic kidney (HEK) 293T cells were purchased from ATCC (American Type Culture Collection).

Protein expression and purification

Overexpression and purification of recombinant human ERp44 (wild type and mutants) and human Ero1 α (hyper active mutant and 2EA mutant) were performed, as described previously²⁹. In all recombinant samples, the histidine tag was cleaved by thrombin (Nacalai Tesque) treatment for overnight before loading onto a SEC column.

For overexpression of ERAP1, the established stable cells were cultured in DMEM supplemented with 5% FCS, 1% penicillin-streptomycin mixed solution (Nacalai Tesque), and 0.5–1 μ g/ml puromycin under 5% CO₂ at 37 °C. For overproduction, cells were cultured in 40 dishes of 15 cm in DMEM supplemented with 5% FCS and 1% penicillin-streptomycin mixed solution for 4 days, and the conditioned medium containing secreted ERAP1 was collected and filtered with a 0.2 μ m filter, and then loaded onto anti-DYDDDK-tag antibody beads (MBL). After washing with 20 CV of washing buffer (50 mM Tris-HCl pH 7.5 300 mM NaCl), the bound proteins were eluted by repeating the 5 min incubations with 1 CV of elution buffer (wash buffer supplemented with 0.1 mg/ml DYKDDDDK peptide). The eluted protein was concentrated using Amicon Ultra-15 centrifugal filters (100 kDa cut-off, Merck-Millipore) with 20 mM Bis Tris pH6.5, 150 mM NaCl, and stored at -80 °C.

SEC analysis of the dissociation of the ERp44-client complexes

Purified client protein (20 μ M ERAP1 or Ero1 α) with equimolar ZnCl₂ was mixed with equimolar ERp44 (20 μ M) and incubated on ice for >30 min to form the Zn-bridged ERp44-client complexes in 20 mM BisTris pH 7.2, 150 mM NaCl. The complexes were diluted (final conc. 10 μ M) and treated with the indicated reagents for >30 min on ice in SEC buffer (20 mM BisTris pH 7.2, 150 mM NaCl). The treated complexes (50 μ l) were loaded on a Superdex 200 increase 10/300 column (Cytiva) equilibrated in SEC buffer using an ÄKTA explorer 100 or 10S systems. Fractions were treated with 20 mM NEM and thereafter analyzed on 7.5% (for ERAP1) or 10% (for Ero1 α) non-reducing SDS-PAGE gels

Cryo-EM sample preparation and data collection

For the preparation of the ERp44-ERAP1 complex, an equimolar mixture (10 μ M) of ERp44, client (ERAP1) and ZnCl₂ was incubated on ice for 30 min and then subjected to a Superdex 200 30/300 increase column. For the preparation of PEG-treated samples, an equimolar mixture (10–12 μ M) of ERp44, client (ERAP1/Ero1 α), and ZnCl₂ was treated with 1 mM methyl-PEG4-NHS ester (Tokyo Chemical Industry) on ice for 1 hour. After quenching the residual PEG with 30 mM Tris, the complex was further purified using a Superdex 200 10/300 increase column. The peak fractions were concentrated with Amicon Ultra 10K.

For grid preparation, 3 μ l of the ERp44-client complex (0.8 mg/ml for ERAP1, 0.5 or 0.9 mg/ml for Ero1 α) was applied to a glow-discharged QuantiFoil R1.2/1.3 300 mesh Au grid for Titan Krios, or a QuantiFoil R1.2/1.3 200 mesh Au grid for CRYO-ARM measurements. For amylamine treatment, 3 μ l aliquots of amylamine (SIGMA) were added to a QuantiFoil grid, and the grid was glow-discharged in the same

manner as usual. The grids were blotted for 3–5 sec and immersed in liquid ethane using Vitrobot Mark IV systems (FEI/Thermo Fisher) operated at 4 °C and 100% humidity.

Cryo-EM measurements of the ERp44-client complexes were performed using CRYO ARM 300II (JEOL ,Tohoku University) operated at 300 kV and equipped with a JEOL in-column Omega energy filter and a Gatan K3 BioQuantum detector; CRYO ARM 200 (JEOL, Kyushu University) operated at 200 kV and equipped with a JEOL in-column Omega energy filter and a Gatan K3 BioQuantum detector; and Titan Krios G4 (Thermo fisher, Okayama University) operated at 300 kV and equipped with an E-CFEG electron source, a post-column mounted Selectris X energy filter and a Falcon 4i camera. Movies were automatically collected using SerialEM⁴³(for CRYO ARM) or EPU (for Krios). The data collection parameters are summarized in Table 1.

Image processing

Image processing was mainly performed using CryoSPARC⁴⁴ V4.1~4.7. Bayesian polishing⁴⁵ was performed by RELION 4.0⁴⁶ with the help of UCSF pyem tool⁴⁷ for data conversion from CryoSPARC to Relion. The details of image processing are summarized in Supplementary Fig. 2, 3, 5, 6 and 7.

For the ERp44-ERAP1 complex, 4500 movies were collected and aligned using beam-induced motion correction implemented in RELION v4.2, followed by contrast transfer function estimation with Patch-based CTF estimation. Rapid Initial processing generated an initial map of the complex, which created templates for template-based autopicking. Particles picked by template-based autopicking were extracted at a pixel size of 3.152 Å, followed by 2D classification. Good classes with clear 2D average images were selected based on similarity to the initial map (reference-based auto-select 2D job). Subsequent ab initio reconstruction and heterogeneous refinements revealed several binding modes of the complex. For particles belonging to “binding mode I,” particles were further selected by ab initio reconstruction and then subjected to homogenous refinement with global and local CTF refinement, Bayesian polishing, and homogenous refinement with global and local CTF refinement. The refined particles were further classified into state1 and state 2 based on two rounds of 3D variability analysis⁴⁸. The final maps of states I and II in binding mode I were refined to 3.75 and 3.87 Å resolutions, respectively. For particles belonging to “binding mode II, particles were re-picked based on the initial mode II map and then subjected to a similar process as mode I. The best particles were selected based on the focused 3D classification in CryoSPARC using a mask covering the ERp44 portion and refined to 4.37 Å resolution.

For the ERp44-ERAP1 complex using UltrAuFoil grids, 3627 movies were collected and aligned using beam-induced motion correction implemented in RELION, followed by contrast transfer function estimation with Patch-based CTF estimation. To collect more different views of the particles, 1457 movies tilted at 15°, 1768 movies tilted at 15°. 1400 movies tilted at 30°, and 1625 movies tilted at 30° were collected. Particles picked by template-based auto-picking using an initial map were extracted at a pixel size of 3.152 Å and subjected to 2D classification, ab initio reconstruction, and heterogeneous

refinements. The best particles were selected and subjected to homogenous refinement with global and local CTF refinements. The particles were further analyzed by 3DVA and intermediated analysis in CryoSPARC using a mask covering the ERp44 portion. The final map of the best class was refined to 3.05 Å resolution.

For the ERp44-Ero1α complex, movies were collected and aligned using beam-induced motion correction with Patch Motion correction in CryoSPARC, followed by contrast transfer function estimation with Patch-based CTF estimation. Initial processing generated cryo-EM maps of two classes of the complexes (binding modes I and II), which were used to train a picking model for Topaz⁴⁹. The best 3D map for binding mode I was obtained from dataset 1 (7150 movies) collected from amylamine-treated grids. Particles picked by Topaz were extracted at a pixel size of 3.312 Å and subjected to 2D classification, ab-initio reconstruction and heterogeneous refinement. The best particles were re-extracted at a pixel size of 1.0212 Å and subjected to two cycles of non-uniform (NU) refinement with global and local CTF refinement, reference-based motion correction, and NU refinement. Although the resolution of the refined map reached 2.9 Å, the map suffered from severe preferred orientation bias, which made it difficult to assign secondary structures to the map. To overcome the orientation bias, the particles with overpopulated viewing directions were removed by Rebalance Orientations job in CryoSPARC, and then the rebalanced particles were refined to 3.66 Å with 3D refinement in combination with blush regularization in Relion 5.0³³.

To further resolve the interface between Ero1α and ERp44, the particles belonging to binding mode I from the three datasets were merged and subjected to 2D classification, NU refinement, and local refinement using a mask covering the ERp44-b' domain and Ero1α. The particles were further classified by focused 3D classification in CryoSPARC on the interface region. The best class particles were subjected to local refinement, orientation rebalance, and 3D refinement with blush regularization. The final cryo-EM map shows an improvement in the local resolution around the interface. Local resolution estimations were performed in Relion.

The best 3D maps for binding mode II were obtained from dataset 4 (10671 movies). Particles picked by Topaz were extracted at a pixel size of 2.912 Å and subjected to 2D classification, ab initio reconstruction, and heterogeneous refinements. The particles in binding mode II were selected and subjected to NU refinement with global and local CTF refinement, reference-based motion correction, and NU refinement. The refined map at a lower level showed extra density next to the b' domain, suggesting the existence of another molecule. The particles were re-extracted in a larger box (a 480-pixel box downsampled in a 120 pixel box) at a pixel size of 2.912 Å and subjected to 2D classification. The particles in the monomers and dimers were subjected to ab initio reconstruction and heterogeneous refinements. The particles in the dimeric complex were further cleaned up with ab initio reconstruction, re-extracted at a pixel size of 0.9693 Å, and refined with NU refinement to a resolution of 4.13 Å. The particles in the monomeric complex were further cleaned up with ab initio reconstruction, re-extracted at a pixel size of 0.8724 Å, and refined with NU refinement and local refinement. The particles were further

classified by focused 3D classification in CryoSPARC on the ERp44 portion. The best particles were further refined with local refinement to 3.90Å resolution.

Model building, refinement

The published crystal structures of metal-free ERp44 (PDB ID:5GU6), Zn-bound ERp44 (PDB ID: 5XWM), ERAP1 (PDB ID: 3QNF), and Ero1α (PDB ID: 3AHQ) were docked in the EM map using Chimera X⁵⁰ or Jiggle Fit in Coot⁵¹. Missing residues in the deposited crystal structures were manually built with Coot⁵¹ using AlphaFold predicted model³⁸. The models were further refined with phenix.real_space_refine⁵² and manual correction with Coot. Structural figures were prepared with PyMOL (<http://www.pymol.org>) and Chimera X. The map reconstruction and model refinement statistics are summarized in Table 1

Declarations

Author contributions

S.W. designed the research and performed almost all experiments, cryo-EM data collection, image processing. M.K., A.M. E.H. performed plasmid construction, sample purification, and dissociation analysis. Y.A. performed plasmid construction and analyzed the data. S.W., S.R. K.I. wrote the manuscript with the help of the other authors. K.I. supervised this work.

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Data availability

The cryo-EM density maps and atomic coordinates have been deposited in the Electron Microscopy Data Bank (EMDB) and the Protein Data Bank under accession codes EMD-69135 and 23OW (ERp44-ERAP1,

mode I, state 1), EMD-69136 and 23OY (ERp44-ERAP1, mode I, state 2), EMD-69141 and 23PE (ERp44-ERAP1 mode II), EMD-69133 and 23OU (ERp44-ERAP1 at high resolution), EMD-69146 and 23PL (ERp44-Ero1 α mode I), EMD-69147 and 23PM (ERp44-Ero1 α mode I, high resolution), EMD-69149 and 23PO (ERp44-Ero1 α mode II monomer), EMD-69146 and 23PL (ERp44-Ero1 α Mode II dimer), respectively. The following atomic coordinates were used in this study: 5GU6 (crystal structures of metal-free ERp44), 5XWM (crystal structure of Zn²⁺-bound ERp44), 3QNF (crystal structure of ERAP1) and 3AHQ (crystal structure of Ero1 α).

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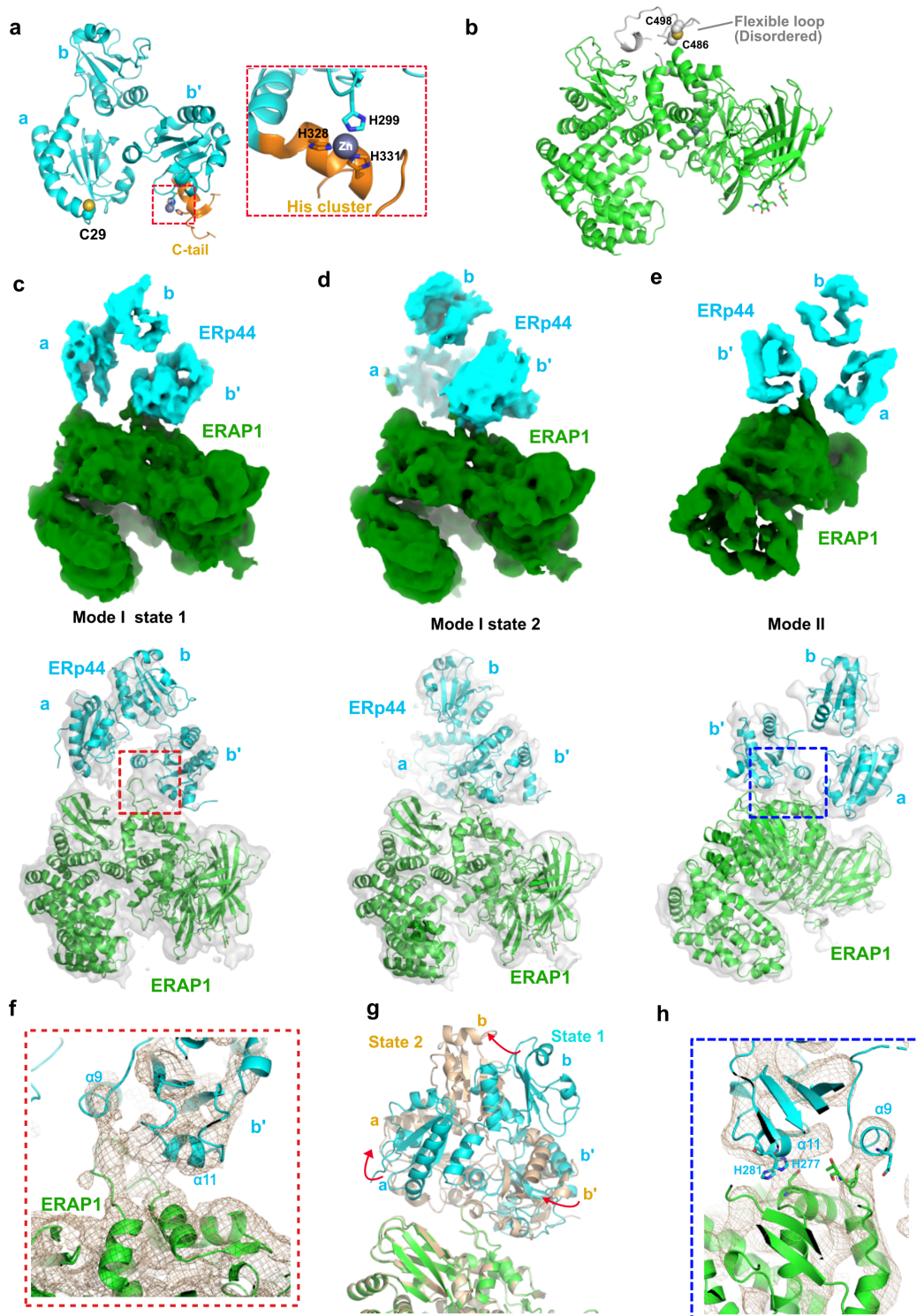
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Table 1

Table 1 is available in the Supplementary Files section.

Figures



Watanabe et al, Fig 1

Figure 1

Overall structure of the ERp44-ERAP1 complex

a, Crystal structure of Zn²⁺-bound ERp44 (PDB ID: 5XWM). The inset highlights a close-up view of the Zn²⁺-binding site formed by the His cluster. The three Trx domains of ERp44 are labeled **a**, **b**, and **b'** from the N-terminus, followed by the C-terminal tail (C-tail) shown in orange.

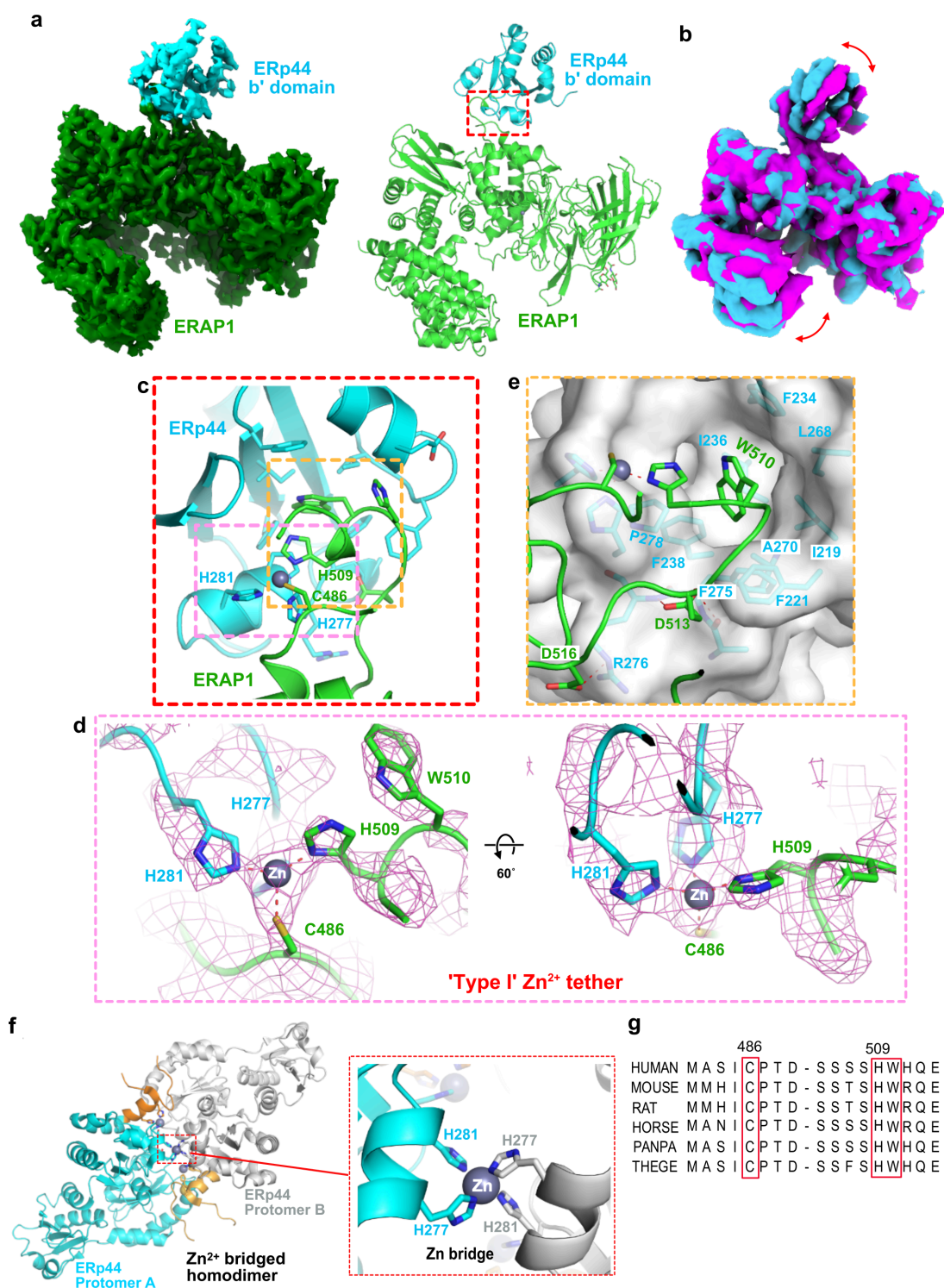
b, Crystal structure of ERAP1 in the open state (PDB ID: 3QNF). The model of the long loop predicted by AlphaFold3, which is structurally disordered in the deposited structure, is shown in gray.

c, d, e Upper panels: Cryo-EM maps of the ERp44-ERAP1 complex in state 1 (**c**), state 2 of binding mode I (**d**), and binding mode II (**e**). ERp44 and ERAP1 are shown in cyan and green, respectively; Lower panels: Ribbon representation of the structure models of the complex overlaid onto the transparent cryo-EM maps.

f, Close-up view of the red dashed box in **c**, overlaid onto the EM density map (brown mesh), showing the interaction between the **b'** domain of ERp44 (cyan) and the flexible loop of ERAP (green) in binding mode I.

g. Comparison of relative positions of ERp44 in state 1 and state 2 of binding mode I with respect to ERAP1.

h, Close-up view of the interface indicated by the blue dashed box in **e**, overlaid onto the EM density map (mesh representation), showing the interaction between the **b'** domain of ERp44 and the flexible loop of the ERAP in binding mode II



Watanabe et al, fig 2

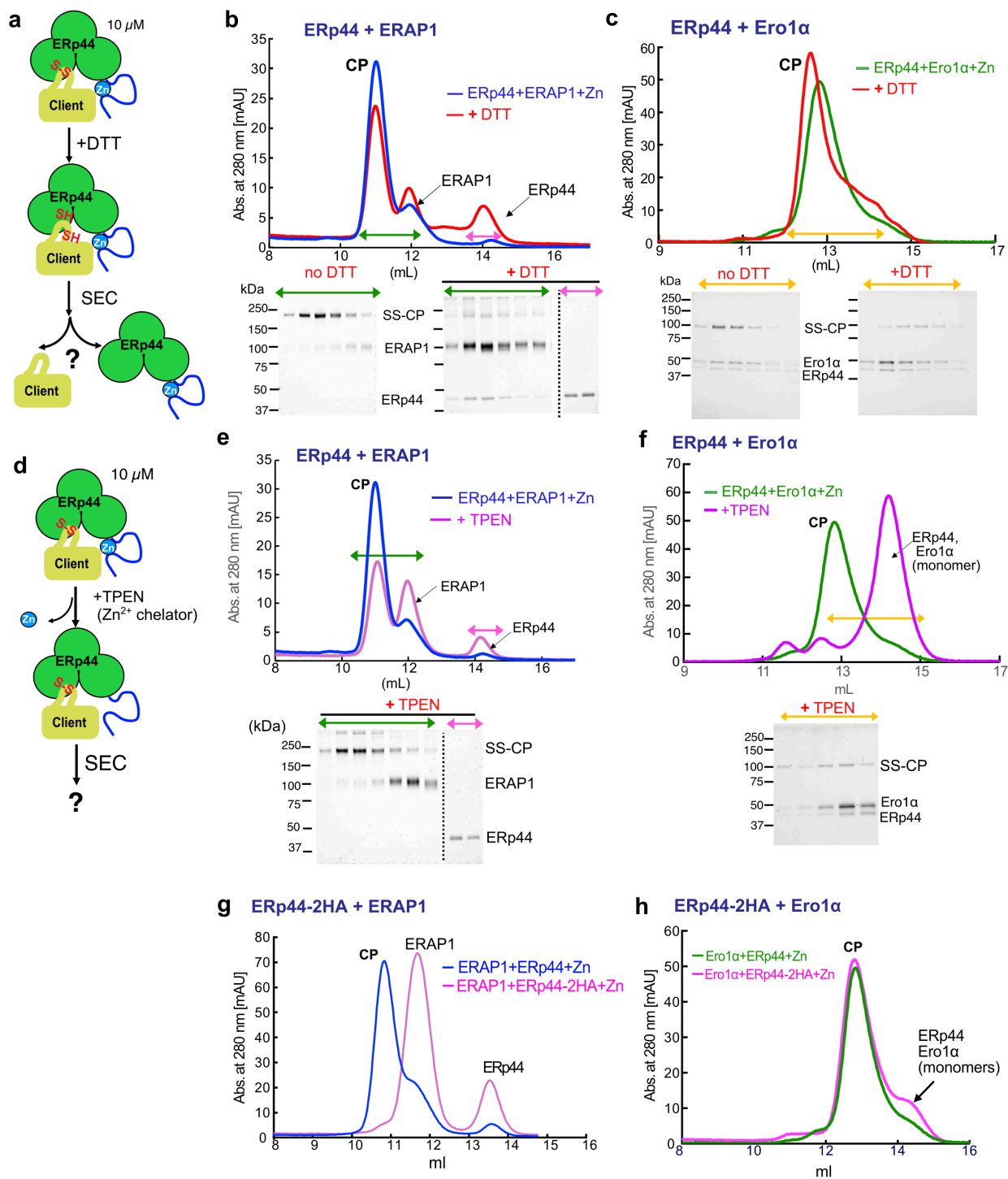
Figure 2

High resolution cryo-EM structure of the ERp44-ERAP1 complex stabilized by type I Zn²⁺ tether

a, High-resolution cryo-EM map (left) and structural model (right) of the ERp44-ERAP complex.

b, First (blue) and last frames (magenta) of the continuous motion of the complex revealed by 3DVA

- c**, Highlighted view of the interaction between ERp44 and ERAP1, indicated by the red dashed box in panel **a**, right.
- d** Close-up views of the interface indicated by the magenta dashed box in **c**, overlaid on the EM density map (magenta mesh), highlighting the type I Zn^{2+} tether formed by His277 and His281 of ERp44 and Cys486 and His509 of ERAP1.
- e**, Close-up view of interface indicated by the yellow dashed box in **c**, highlighting the hydrophobic pocket of the ERp44 **b'** domain (surface representation) that accommodates Trp510 of ERAP1.
- f**, Crystal structure of the Zn^{2+} -bridged ERp44 dimer (PDB ID: 5XWM). The inset shows a close-up view of the Zn^{2+} -bridge formed by two pairs of His277 and His281 at the center of the homodimer.
- g**, Conservation of residues involved in the type I Zn^{2+} tether among ERAP1 orthologues.



Watanabe et al, Fig 3

Figure 3

Dissociation assay for the ERp44-client complex

a, Schematic of the dissociation assay for the DTT-treated ERp44-client complex.

- b**, SEC elution profiles of the non-treated (control, blue) and DTT-treated (red) ERp44-ERAP1 complex (upper panel). The peaks corresponding to the complex (CP), isolated ERAP1, and isolated ERp44 are indicated. The fractions indicated by green and magenta arrows were analyzed by non-reducing SDS-PAGE (lower panel).
- c**, SEC elution profiles of the non-treated (green) and DTT-treated (red) ERp44-Ero1 α complex (upper panel). The fractions indicated by yellow arrow were analyzed by non-reducing SDS-PAGE (lower panel).
- d**, Schematic of the dissociation assay for the TPEN-treated ERp44-client complex
- e**, SEC elution profiles of the non-treated (control, blue) and TPEN-treated ERp44-ERAP1 complex (magenta) (upper panel). The fractions indicated by green and magenta arrows were analyzed by non-reducing SDS-PAGE (lower panel)
- f**, SEC elution profiles of the non-treated (control, green) and TPEN-treated ERp44-Ero1 α complex (magenta) (upper panel). The fractions indicated by yellow arrow were analyzed by non-reducing SDS-PAGE (lower panel).
- g**, SEC elution profiles of the mixture of ERAP1 and ERp44 WT (blue) or the ERp44-H277A/H281A (2HA) mutant (magenta) in the presence of Zn²⁺.
- h** SEC elution profiles of the mixture of Ero1 α and wild-type ERp44 (green) or the ERp44 2HA mutant (magenta) in the presence of Zn²⁺.

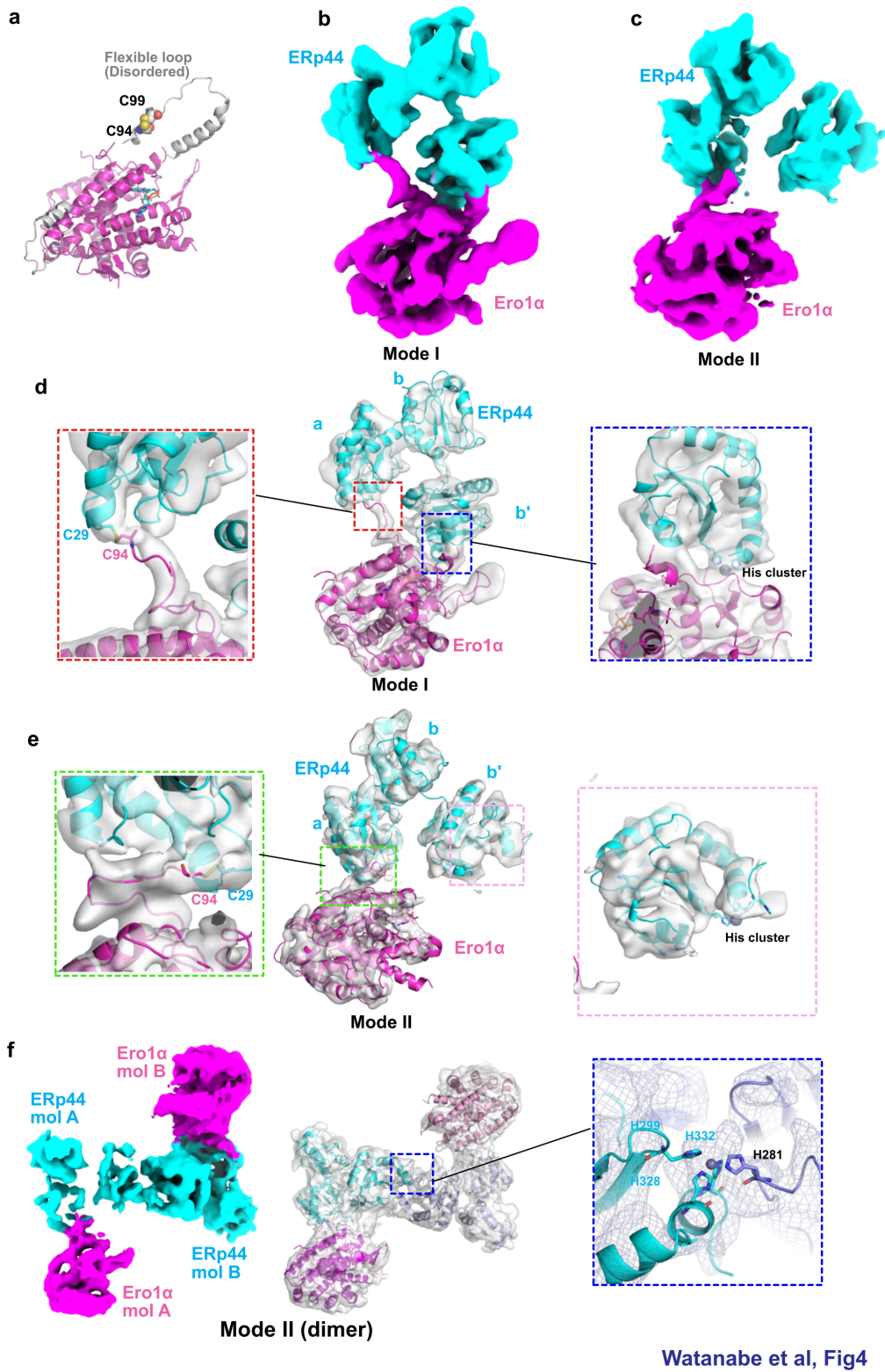


Figure 4

Overall structure of the ERp44-Ero1α complex

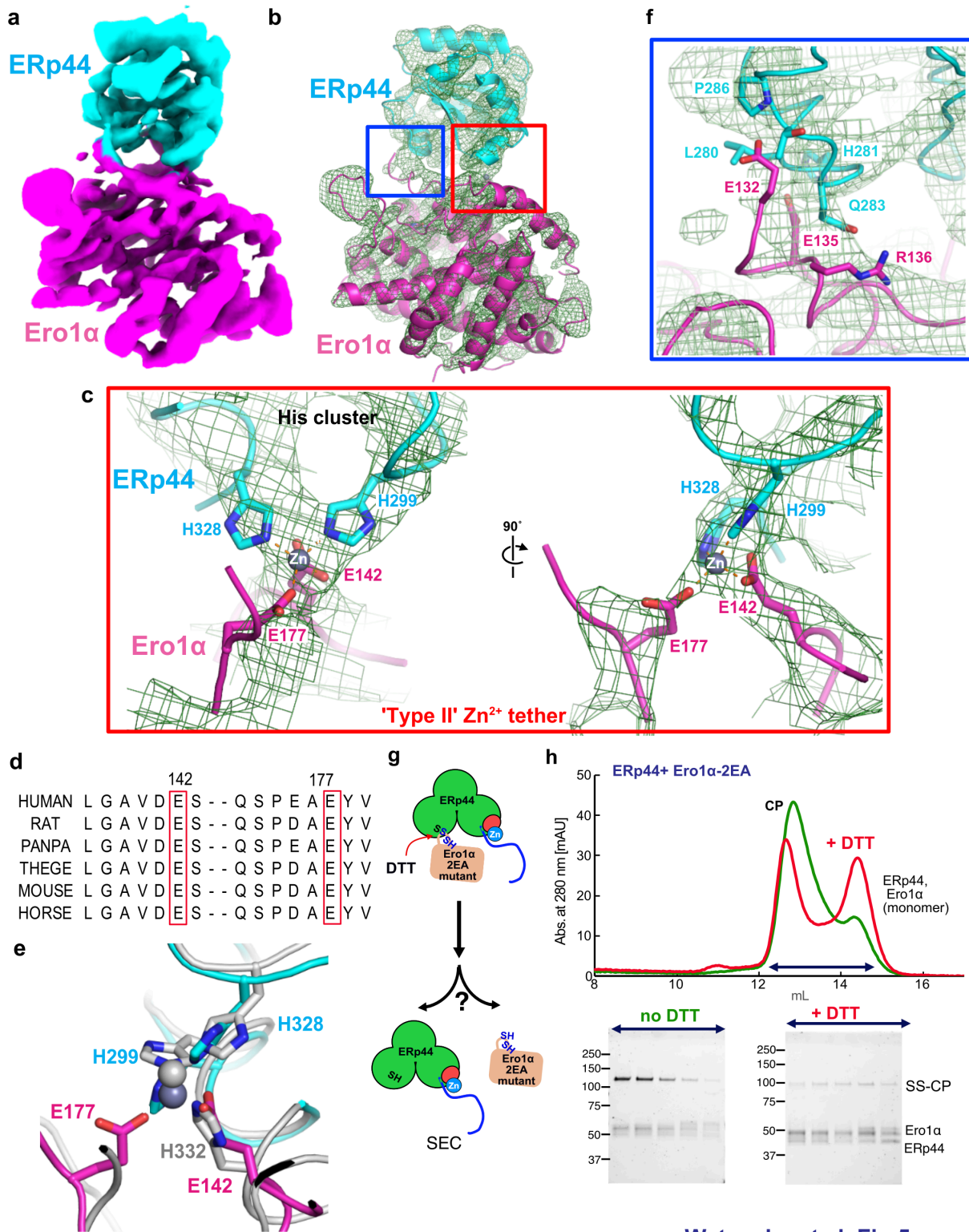
a, Crystal structure of human Ero1α in the hyperactive form (PDB ID: 3AHQ). The model of the long loop predicted by AlphaFold3, which is structurally disordered in the deposited structure, is shown in gray.

b, c, Cryo-EM maps of the ERp44-Ero1 α complex in binding modes I (b) and II (c). Densities of ERp44 and ERAP1 are shown in cyan and green, respectively.

d, Structural model of the ERp44-Ero1 α complex in binding mode I, overlaid onto the transparent EM map. The insets show close-up views of the interface indicated by red and blue dashed boxes.

e, Structural model of the binding mode II of the ERp44-Ero1 α complex, overlaid onto the transparent EM map. The insets show close-up views of the interfaces and his cluster indicated by light-green and pink dashed boxes.

f, Cryo-EM map and structural model of the dimer of the ERp44-Ero1 α heterodimer in binding mode II (c). The inset shows a close-up view of the possible Zn²⁺-mediated interaction between the His cluster of one protomer and His288 of another protomer.



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Figure 5

Type II Zn^{2+} tether at the ERp44-Ero1α interface

a, Improved cryo-EM map of the ERp44–Ero1α complex. Note that density corresponding to ERp44 is observed only for the **b'** domain after local refinement.

- b**, Model of the complex between ERp44 **b'** domain and Ero1 α overlaid onto the improved EM density map (green mesh).
- c**, Close-up view of the interface indicated by the red box in **b**, overlaid onto the EM density map (green mesh), highlighting the type II Zn²⁺ tether formed by His299 and His328 of ERp44 and Glu142 and Glu177 of Ero1 α .
- d**, Conservation of Glu142 and Glu177 residues among Ero1 α orthologues.
- e**, Conformational comparison of the His cluster of ERp44 in complex with Ero1 α and in the client-free, Zn²⁺-bound form (white, PDB ID: 5XWM).
- f**, Close-up view of the interface indicated by the blue box in **b**, overlaid onto the EM density map.
- g**, Schematic of the dissociation assay for the complex between ERp44 and the Ero1 α E142A/E177A (2EA) mutant
- h**, SEC elution profiles of the complex between ERp44 and Ero1 α 2EA mutant with (red) and without (green) DTT pretreatment (upper panel). The fractions indicated by a dark blue arrow were analyzed by non-reducing SDS-PAGE (lower panel).

b, SEC elution profiles of the ERp44-ERAP1 complex pretreated with 10 mM GSH and eluted in buffers containing 10 mM GSH (red) or lacking GSH (blue) (upper panel). The fractions indicated by blue, green and red arrows were analyzed by non-reducing SDS-PAGE (lower panel).

c, SEC elution profiles of the ERp44-Ero1 α complex pretreated with 10 mM GSH and eluted in buffers containing 10 mM GSH (red) or lacking GSH (blue) (upper panel). The SEC elution profile of the TPEN-treated sample is shown in purple. The fractions indicated by cyan and green arrows were analyzed by non-reducing SDS-PAGE (lower panel).

d, Schematic illustration of the Zn²⁺-mediated client binding and release of ERp44 in the secretory pathway (see text and Supplementary Figs. 9b and 10). The histidine cluster and H277/H281 pair of ERp44 and cysteine residues involved in the mixed disulfide bonds are indicated by red, magenta, and orange circles, respectively.

Supplementary Files

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