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A disulphide bond in the E2 enzyme Pex4p modulates ubiquitin-conjugating activity

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The ubiquitin-conjugating enzyme Pex4p together with its binding partner, the peroxisomal membrane protein Pex22p, co-ordinates cysteine-dependent ubiquitination of the cycling receptor protein Pex5p. Unusually for an ubiquitin-conjugating enzyme, *Saccharomyces cerevisiae* Pex4p can form a disulphide bond between the cysteine residues at positions 105 and 146. We found that mutating the disulphide forming cysteine residues in Pex4p to serines does not disturb the secondary structure of the protein but does reduce the *in vitro* activity of Pex4p. From the crystal structure of Pex4p C105S, C146S in complex with the soluble domain of Pex22p, we observe a narrowing of the active site cleft, caused by loss of the disulphide bond. This modification of the active site microenvironment is likely to restrict access of ubiquitin to the active site cysteine, modulating Pex4p activity. Finally, based on sequence and structural alignments, we have identified other ubiquitin-conjugating enzymes that may contain disulphide bonds.

In the yeast *Saccharomyces cerevisiae* the import of enzymes into the peroxisomal matrix requires the action of the peroxisome associated ubiquitin-conjugating enzyme (UBC or E2) Pex4p[1]. Pex4p's function is to ubiquitinate Pex5p, the cycling receptor required for import of enzymes displaying a peroxisomal targeting signal (PTS) type one[2,3]. This modification allows Pex5p to be removed from the peroxisomal membrane, in order to take part in another round of import[2,4,5]. We recently solved the structure of *Saccharomyces cerevisiae* Pex4p in complex with the soluble domain of the peroxisomal membrane protein Pex22p[6]. Although Pex4p is an active E2, it requires Pex22p to modify Pex5p. Binding of Pex22p allows Pex4p to associate with the peroxisomal membrane[7], stimulates the transfer of ubiquitin (Ub) to a substrate and is likely to play a role in recruiting the E3 ligase required for Pex5p modification[6].

During our studies, we observed that Pex4p, when in complex with Pex22p, contains an unexpected disulphide bond, formed between the cysteine residues at positions 105 and 146 (Figure 1a). Cysteine 105 in Pex4p is adjacent to the conserved asparagine residue that catalyses isopeptide bond formation and consequently Ub chain formation[8]. A cysteine at this position represents a unique variation on the highly conserved histidine-proline-asparagine motif found in most E2 enzymes (Figure S1). Disulphide bonds can stimulate protein folding, by restricting the mobility of certain sections of the protein or by providing a local hydrophobic core around which other hydrophobic residues can condense[9]. Disulphide bond formation in eukaryotic cells almost exclusively occurs in the oxidising environment of the endoplasmic reticulum (ER) and disulphides are generally not observed in the cytosol, due to its reducing potential[10]. However disulphide bonds can be found outside the ER and proteins that contain them are often involved in sensing the redox potential of the cytosol[11,12].

In this study, we confirm the presence of a disulphide bond in Pex4p and show that disrupting disulphide bond formation reduces the *in vitro* activity of Pex4p, without causing major structural changes to the protein. Based on the crystal structure of the disulphide bond mutant form of Pex4p in complex with Pex22p, we propose a basis for the loss of activity seen due to the absence of the disulphide bond. Furthermore, we show that members of the Ube2E family of ubiquitin-conjugating enzymes may also contain disulphide bonds in similar positions.

Results

Pex4p contains a disulphide bond in solution. To address the potential role of the disulphide bond in Pex4p activity, we first assessed whether mutating cysteine 105 and 146 to serines may disturb the secondary structure of Pex4p using circular dichroism (CD) spectropolarimetry (Supplementary Figure S2). However, the secondary
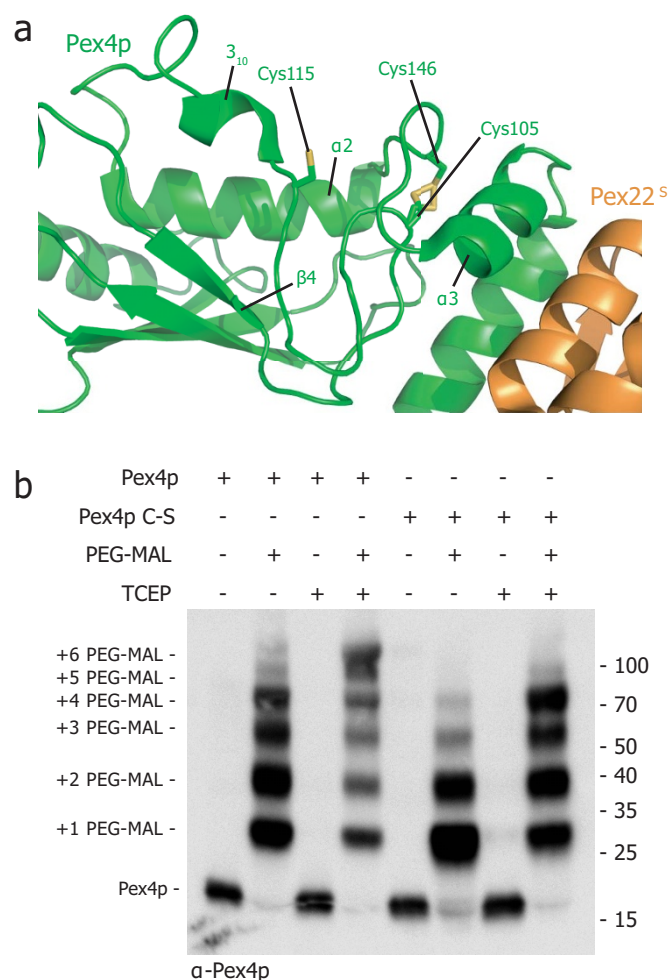
Figure 1 | The Pex4p-Pex22[S] complex contains a disulphide bond.
(a) Cartoon representation of the Pex4p (green) Pex22[S] (orange) structure (PDB 2y9m), showing the disulphide bond (Cys105-Cys146) and active site cysteine (Cys115) as well as surrounding secondary structure elements. (b) Pex4p in solution contains four free thiol groups. Wild-type or C105S,C146S (C–S) forms of Pex4p were denatured with urea and treated with the thiol-modifying reagent PEG-MAL 5000. Equal amounts (containing approximately 2.5 μg of Pex4p) of the reaction mixture were subjected to SDS-PAGE, western blotting and staining with anti-Pex4p antibodies. Under non-reducing conditions (TCEP -), four of the six cysteine residues present in Pex4p can be readily modified, whereas under reducing conditions (TCEP +), all six can be modified. When the cysteine residues proposed to form the disulphide bond are mutated to serines, only four species are formed after PEG-MAL 5000 treatment, under both reducing and non-reducing conditions.

structure of Pex4p C105S,C146S is comparable with that of wild-type Pex4p, suggesting that the function of the disulphide bond in Pex4p is unlikely to be in aiding protein folding. Next, we determined whether the disulphide bond is indeed present in Pex4p in solution, to rule out the possibility that it is a crystallisation artefact. Pex4p was treated with polyethylene glycol maleimide (PEG-MAL) 5000 and the protein was analysed by SDS-PAGE (Figure 1b). PEG-MAL specifically reacts with free thiol groups and does not react with cysteine residues present in disulphides[13]. Attachment of a single PEG-MAL moiety to a protein results in an increased molecular weight of around 5000 Da. However, the attachment of several PEG-MAL moieties can significantly decrease mobility of proteins on SDS-PAGE, resulting in a higher molecular weight than expected for each species. Pex4p contains six cysteines, two of which are

involved in disulphide bond formation. Addition of PEG-MAL to denatured Pex4p resulted in the formation of four major and two minor anti-Pex4p antibody-reactive species (Figure 1b). To confirm that indeed all six cysteines in Pex4p can be modified by PEG-MAL under conditions where disulphide bridge formation is disfavoured, we treated Pex4p with the reducing agent tris-(2-carboxyethyl)-phosphine (TCEP), followed by PEG-MAL addition. This time, Pex-4p was modified by up to six PEG-MAL moieties, indicating that under reducing conditions two additional cysteines in Pex4p become readily accessible to PEG-MAL modification. Furthermore, addition of PEG-MAL to Pex4p lacking the disulphide-bonded cysteines observed in the crystal structure (Pex4p C105S,C146S), irrespective of whether TCEP treatment was performed or not, gave an almost identical result as that observed for wild-type Pex4p in the absence of TCEP, i.e. the formation of four anti-Pex4p reactive species (Figure 1b). To confirm these observations with a separate, more quantitative technique, we treated denatured Pex4p with iodoaceta-mide (IA) and performed mass spectrometry (Supplementary Figure S3). As with PEG-MAL, IA specifically reacts with free thiol groups[14] and attachment of an IA moiety to a protein increases its molecular weight by 58 Da. According to our mass spectrometry analysis Pex4p, under reducing or non-reducing conditions, has a molecular weight of around 19576 Da (Supplementary Figure S3). After IA addition, the molecular weight of Pex4p increased by around 233 $+/-$ 6 Da, equivalent to the attachment of 4.0 IA moieties. However, if Pex4p is reduced with TCEP prior to IA treatment, the molecular weight increase is around 343 $+/-$ 9 Da, equivalent to the addition of 5.9 IA moieties. Therefore, our data indicate that the cysteines at positions 105 and 146 in Pex4p do indeed form a disulphide bond.

Pex4p lacking a disulphide bond exhibits reduced activity. Having provided evidence that the disulphide bond is present in the Pex4p used in our previous assays[6,15] we asked whether this novel feature would play a role in Pex4p activity. To this end, we assessed the effect of the C105S,C146S mutations on Pex4p's activity in an E2 self-ubiquitination reaction (Figure 2). Although this assay does not probe attachment of Ub to Pex4p's substrate Pex5p, we have successfully used the method to gain insight into Pex4p activity[6,15]. We observed that Pex4p C105S,C146S self-ubiquitination was considerably reduced compared to the wild-type protein (Figure 2b), irrespective of whether the soluble region of Pex22p (Pex22[S]) was present. To gauge possible effects of the C105S,C146S mutations on Pex4p's ability to accept Ub from the E1 enzyme, we performed E2 loading experiments (Figure 2c). The ability of Pex4p C105S,C146S to accept Ub was slightly reduced when compared to the wild-type protein, although after 10 min a significant amount of thioester-linked Ub-Pex4p was produced. Taken together, these data suggest an important role for the disulphide bond in Pex4p's E2 activity, given that its loss disturbs the enzymes ability to transfer Ub to a substrate, as assessed by the auto-ubiquitination assays presented in Figure 2.

Structural basis for disulphide bond-modulated Pex4p activity. To gain insight into the mechanism by which the disulphide bond modulates ubiquitin conjugating activity, we determined the three-dimensional structure of Pex4 C105S,C146S in complex with Pex22[S] at 3.23 Å resolution (Figure 3, Figure S4, Table I). The structure is highly similar to the wild-type complex structure (Figure S4a), although the limited resolution does not allow for a highly accurate description of the mutant complex. Nevertheless, a shift in the positioning of the loop between α-helices α2 and α3, the loop where cysteine 146 is normally present (Figure 3a) can be seen. This loop, which forms one side of the cleft where the active site cysteine is found, is fixed by the disulphide bond to the loop region between β-strand β4 and the 3₁₀ helix, where cysteine 105 is present (Figure 1a). Disruption of the disulphide bond therefore causes changes to the active site environment (Figures 3). Furthermore, the density of the
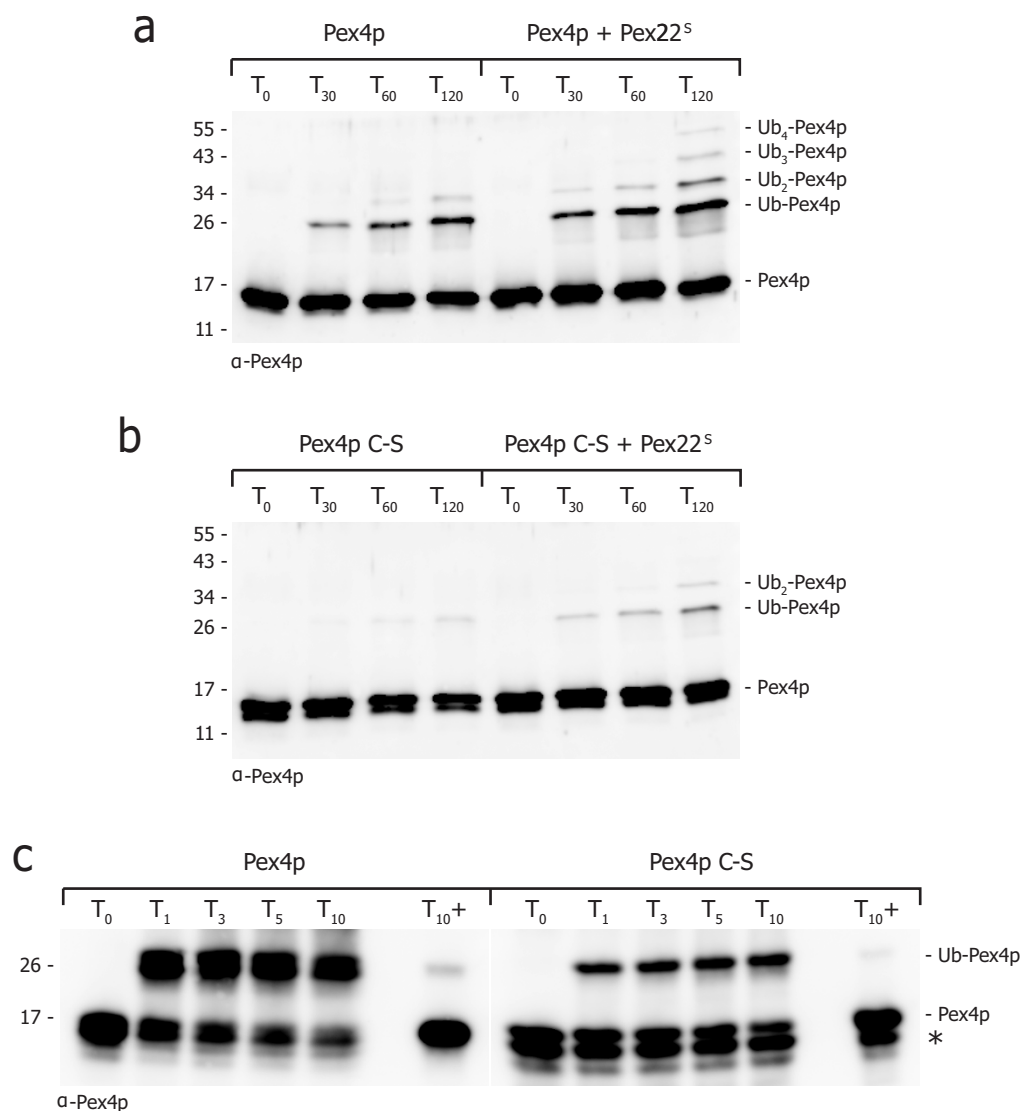
Figure 2 | Disruption of the disulphide bond reduces Pex4p activity. *In vitro* ubiquitination reactions with wild-type (a) or Pex4p C105S,C146S (b) performed in the absence (left panels) or presence (right panels) of Pex22^S. (c) E2 loading experiments with wild-type (left panel) or C105S,C146S (right panel) forms of Pex4p. Samples were resolved by non-reducing SDS-PAGE. (+) Indicates the addition of the reducing agent β-mercaptoethanol, to assess the level of E2 self-ubiquitination after 10 min. (*) Indicates a breakdown product of Pex4p.

side chains in the α2-3 loop is not well defined (Figure S4b), which suggests that this loop becomes more flexible in the absence of the disulphide bond. Superimposition of our structures with that of the SUMO E2 Ube2I poised to transfer SUMO to a substrate[16] shows that the C-terminus of SUMO accesses the active site of Ube2I through the cleft formed by the α2-3 loop (Figure 3b). Loss of the disulphide bond is likely to restrict access of Ub and/or the substrate to the active site cysteine of Pex4p (Figure 3 c and d), affecting the ability of the E2 to transfer Ub to a substrate.

Discussion

Here, we report on a novel feature present in the ubiquitin-conjugating enzyme Pex4p, namely a disulphide bond, formed between cysteines 105 and 146. To the best of our knowledge, this is the first example of an E2 containing a disulphide bond. Indeed, even other members of the Pex4p family of E2 enzymes lack such a unique feature (Supplementary Figure S1). Pex4p from the yeast *Candida glabrata* does contain a histidine-cysteine-asparagine motif (Supplementary Figure S1), but a cysteine that corresponds

to cysteine 146 in *S. cerevisiae* Pex4p is absent. Whether this would suggest that other Pex4p proteins could have lost this feature over time is open to speculation. Several members of the Ube2E family of E2s also contain a histidine-cysteine-asparagine motif (Supplementary Figure S1). Interestingly, Plafker and co-workers recently demonstrated a novel role for this motif in the function of mouse Ube2E3 (UbcM2[17]). They reported that under oxidising conditions, the cysteine residue undergoes post-translational modification, possibly alkylation, which in turn promotes the association of UbcM2 with Nrf2, a transcription factor involved in reactive oxygen species defence[18]. These observations lead the authors to describe the histidine-cysteine-asparagine motif as a redox sensor. Although we have not assessed the role of the disulphide bond *in vivo*, we believe that the disulphide bond in Pex4p could also act as a redox sensor. Since Pex4p functions at the peroxisomal membrane, it can be reasoned that fully active Pex4p in the cytosol would be undesirable. We speculate that the disulphide bond in Pex4p may function in keeping Pex4p in an inhibited state should an excess of Pex4p build up in the cytosol.
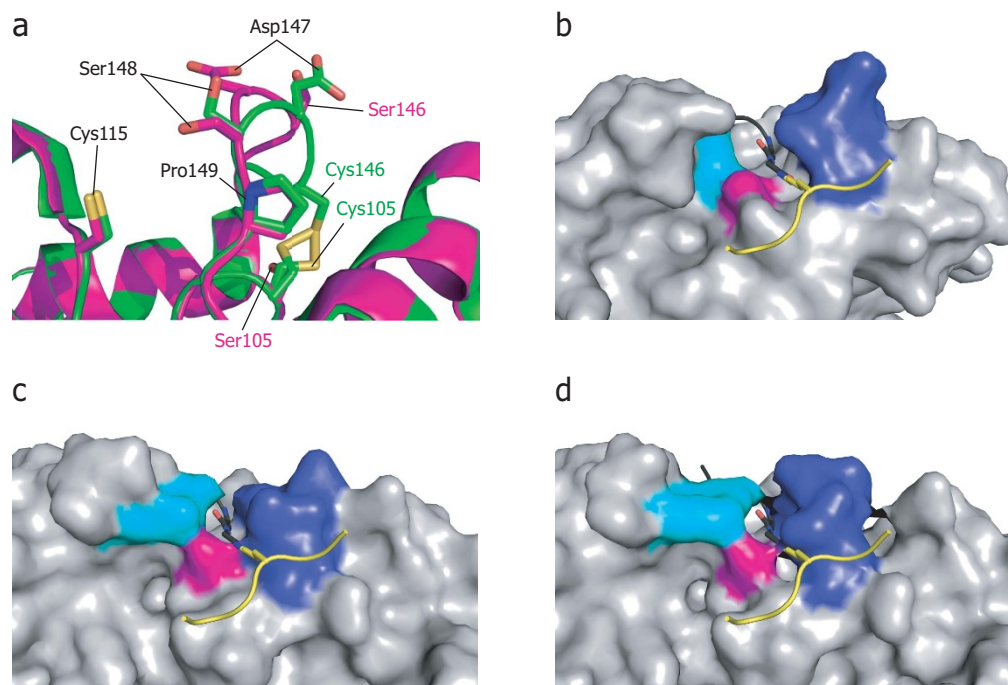
Figure 3 | Crystal structure of Pex4p C105S,C146S in complex with Pex22ˢ. (a) Superimposition of the Pex4p wild-type (green) and C105S,C146S (magenta) structures. Residues specific to the wild-type and C105S,C146S structures are shown in green and magenta, respectively. (b) Surface representation (grey) of Ube2I (PBD 1z5s) poised to transfer the Ub like protein SUMO (black) to a lysine in its substrate RanGAP1 (yellow), indicating the active site (magenta) cleft formed by the 3_{10} helix (cyan) and the α2-3 loop (blue). (c and d) Superimposition of the C-terminal region of SUMO and the target lysine of RanGAP1 (shown in stick representation, colours as in b) onto the structures of wild-type (c) and C105S,C146S (d) Pex4p, indicating the active site (magenta), 3_{10} helix (cyan) and α2-3 loop (blue).

Following on from this, future studies should be aimed at confirming whether the disulphide bond in indeed present *in vivo* and at what step disulphide bond formation occurs.

Our results suggest that the disulphide bond plays a role in controlling the activity of Pex4p. The disulphide bond effectively pins the α2-3 loop to the β4-3_{10} helix loop region (Figure 1a), locking the active site cleft in an open position (Figure 3c). Loss of the disulphide bond leads to an increase in the flexibility of the α2-3 loop, which in turn alters the conformation of the active site cleft (Figure 3d). Reports have indicated the important role residues in the α2-3 loop play in E2 activity and specificity, including regulation of the pKa value of the active site cysteine[19], determining site-specific linkage during Ub chain formation[20,21] and modulation of the type of substrate residue to which Ub is attached[22]. According to those findings, not only are the individual residues themselves important, but their spatial arrangement also plays a crucial role in determining activity and specificity. A repositioning of the α2-3 loop would compromise the spatial arrangement of the active site micro-environment of Pex4p, modulating the activity of Pex4p.

Intriguingly, many members of the Ube2E family from different organisms contain not only a histidine-cysteine-asparagine motif but also a cysteine in the α2-3 loop (Supplementary Figures S1 and S5). The structures of two members of this family have been solved (Ube2E1 and 2 from humans, PBD codes 3BZH and 1Y6L) and while the two cysteines do not form a disulphide bond in these structures, they are in close enough proximity to allow disulphide bond formation to occur (Supplementary Figure S5). It is worthy to note that the α2-3 loop in these structures is in close proximity to the active site cysteine, in a somewhat similar manner to the α2-3 loop in our Pex4p C-S mutant structure (Supplementary Figure S5b). Whether this may be caused by the loss of the putative disulphide bond present in Ube2E1/Ube2E2 during the purification/crystallisation procedure

is open to debate. Taken together, we hypothesise that the activity profile of a subset of E2 enzymes with a histidine-cysteine-asparagine motif and a cysteine in the α2-3 loop could be altered by disulphide bond-induced conformational changes around the active site cleft.

Methods

Strains, plasmids and protein preparation. Plasmids encoding for wild type His₆-GST Pex4$^{15–183}$ (pCW187) and His₆-GST Pex22ˢ (pCW218) are described in[6]. The plasmid encoding His₆-GST Pex4$^{15–183}$ C105S,C146S was constructed using the Quikchange™ site directed mutagenesis kit (Stratagene) using pCW187 as template. All proteins were expressed and purified as described in[15].

Thiol-specific modification of Pex4p. Pex4p (10 μM in 25 mM Tris pH 7.5, 75 mM NaCl) was denatured with 4 M Urea at 30°C in the presence or absence of 0.4 mM tris-(2-carboxyethyl)-phosphine (TCEP) for 30 min. Samples were then treated with either 4 mM methoxy polyethylene glycol maleimide, MW 5000 (PEG-MAL, Jenkem) or 10 mM iodoacetamide (IA, Sigma) for 30 min at 30°C. PEG-MAL reactions were quenched by the addition of loading buffer containing 50 mM dithiothreitol (DTT), subjected to SDS-PAGE, Western blotting and staining with anti-Pex4p antibodies (rabbit, polyclonal). IA reactions were mixed with trifluoroacetic acid (TFA) to a final concentration of 0.1% and then purified using a C:18 ZipTip (Millipore). Samples were eluted from the ZipTip in 50% acetonitrile, 0.1% TFA and subjected to matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry using a Voyager-DE STR (Applied Biosystems). The data presented represent the mean +/− SD of three separate measurements.

In vitro ubiquitination assays. *In vitro* ubiquitination reactions were performed as described in[15] and contained 25 mM Tris pH 8.1, 75 mM NaCl, 5 mM MgCl₂, 5 mM ATP. Protein concentrations were as follows: 0.2 μM rabbit E1 enzyme, 11 μM Ub (Sigma), 1.2 μM Pex4p, 1.7 μM Pex22ˢ. E2 loading experiments[6] contained 25 mM Tris pH 7.5, 75 mM NaCl, 5 mM MgCl₂, 5 mM ATP and 0.27 μM E1, 29 μM Ub, 8.3 μM Pex4p. Reactions were initiated by the addition of ATP and incubated at 28°C with gentle shaking. Samples were taken at the time points mentioned, treated with loading buffer (without reducing agent for E2 loading experiments) and subjected to SDS–PAGE. Western blots were probed with anti-Pex4p antibodies.

Crystallisation, data collection and structure determination. Crystals of the mutant complex were obtained using the same conditions as for the wild-type complex (8.0%

Table I | Data collection and refinement statistics

Data collection	
Space group	C2
Cell dimensions	
a, b, c (Å)	138.8, 42.8, 60.5
α, β, γ (°)	90.0, 100.1, 90.0
Resolution range (Å)	19.86–3.23 (3.4–3.23)#
R_{merge} (%)	13.8 (48.7)
$I/\sigma I$	3.5 (1.8)
Completeness (%)	96.9 (96.7)
Redundancy	2.3 (2.1)
Refinement	
Resolution (Å)	17.28–3.23
No. Reflections	5353
$R_{work}/R_{free}$$	20.3/26.4
No. of atoms in the asymmetric unit	
Protein	2191
Ligand	24
Solvent	7
B-factors	
Protein	47.3
Ligand	66.8
Solvent	27.9
R.m.s. deviations	
Bond lengths (Å)	0.007
Bond angles (°)	1.07
MolProbity validation	
Clashscore (all atom contacts)	3.16 (100th percentile)
Poor rotamers (%)	2.48%
Ramachandran (%)	
Favoured	97.11
Allowed	100
Disallowed	0
C deviations > 0.25 Å	0
MolProbity score	1.57 (100th percentile)
Residues with bad bonds (%)	0
Residues with bad angles (%)	0

#Values in parentheses are for the highest resolution shell.
$R_{free} was calculated with 4.5% of the reflections excluded from the refinement

(V/V) Ethylene glycol, 14.0% (W/V) Polyethylene glycol, 0.1 M HEPES pH 7.0[5]) with the complex at a concentration of 10 mg/ml. Data were collected at a wavelength of 0.812 nm at 100 K on beamline X13 of the EMBL/DESY, Hamburg, processed using XDS[23] and COMBAT and scaled with SCALA[24]. The structure was solved by molecular replacement with MOLREP[25] using the wild-type Pex4p-Pex22p complex structure (PDB code 2y9m) as search model. To avoid model bias, residues 105 and 143–151 from Pex4p were removed from the template and built in manually during the refinement process. Manual model manipulation and solvent incorporation was performed with COOT[26] and refinement was carried out using REFMAC[27]. Model quality was assessed with Molprobity[28]. The final model has 97.11% of the residues in favoured regions and 100% in allowed regions of the Ramachandran plot. Structure representations were made with PyMOL (www.pymol.org). The coordinates and structure factors of the mutant complex have been deposited in the protein data bank (PDB) under the accession code 4bwf.

Circular dichroism (CD) spectropolarimetry measurements. CD measurements were performed at 20°C in 10 mM potassium phosphate, 150 mM NaF, pH 7.4, as described in[6], with individual proteins at a concentration of 10 μM and complexes at 5 μM. Spectra were background subtracted and converted to mean residue ellipticity and represent the mean (+/− SD at 222, 208 and 190 nm) of three separate measurements.

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Acknowledgements

Author contributions

C.W., B.D. and M.W. designed the study; C.W. made the clones, expressed and purified the proteins, performed CD measurements, performed thiol modification experiments, crystallised the complex, collected the X-ray data and solved the structure; M.B. performed *in-vitro* ubiquitination assays; W.A.S. analysed and processed the CD data; M.W. and B.D. supervised the project; C.W. wrote the paper, with contributions from all authors.

Additional information