The peroxisomal matrix protein translocon is a large cavity-forming protein assembly into which PEX5 protein enters to release its cargo.

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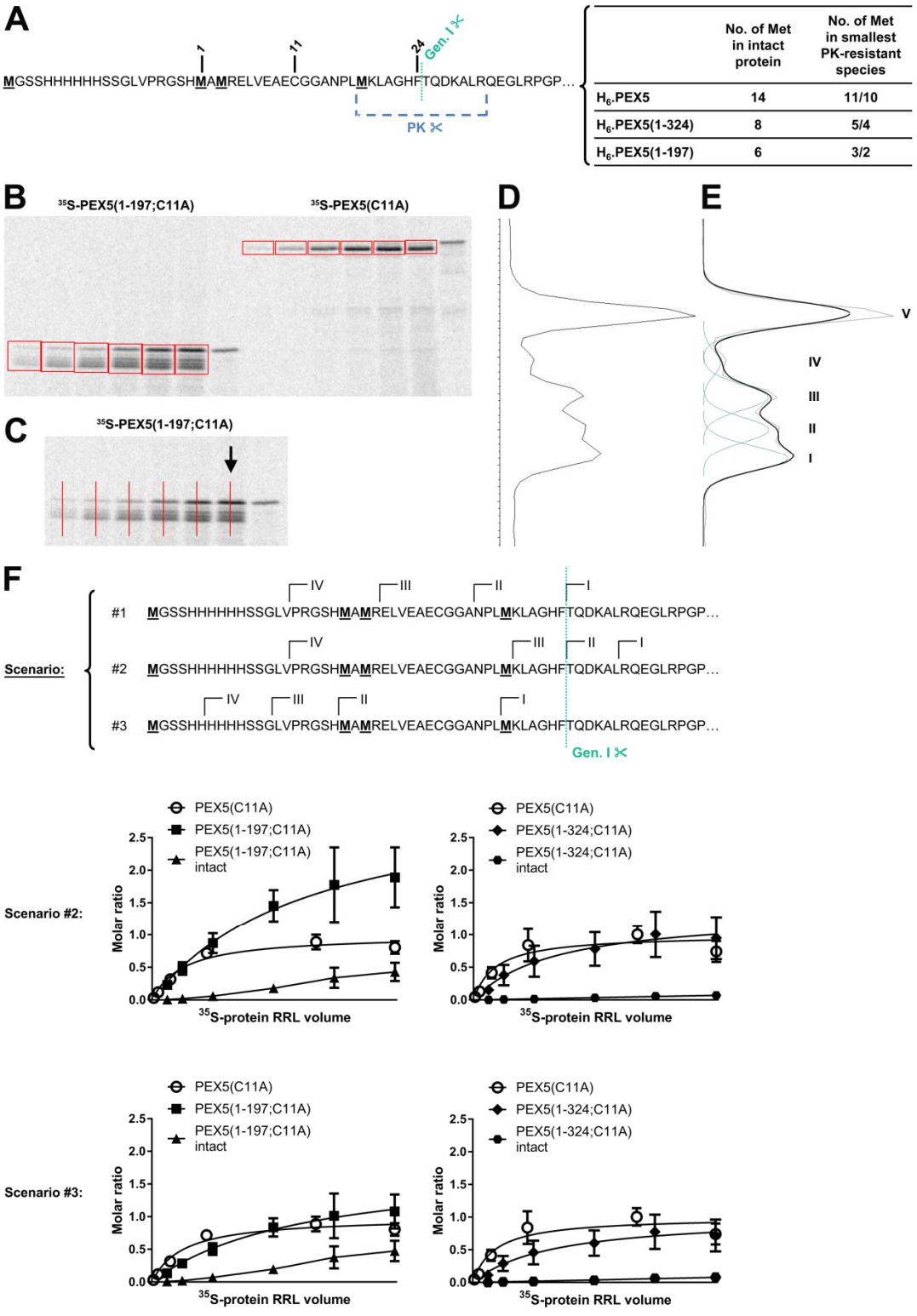
Supplemental material includes:

TABLE S1. Oligonucleotides used in this study.

Plasmid	**Sequence 5' - 3'**
pET-28-PEX5	Fw:GCGAACTGCATATGGCAATGCGGGAGCTGG Rv:GCGTAATTAAGCTTGGCTGCAGGTC
pET-28-PEX5(1-125;C11A)	Fw:GATGCGTCATATGGCAATGCGGGAGCTGGT Rv:GATCGCAAGCTTTCAAGCTGCAAGAAACTCCTG
pET-28-PEX5(1-197;C11A)	Fw:CTCGATCCCGCGAAATTAATACGACTC Rv:CGCCAAGCTTTTACGTGTGCTGCAGATCCTCCTC
pET-23-PEX5(ΔN137)	Fw:GGAACGCATATGACTGACTGGTCCCAAGAATTCATCTC Rv:GATAACGTCGACTCACTGGGGCAGGCCAAACATAG
"for expression PCR" PEX5(1-197;C11A)	Fw:GCCCAATACGCAAACCGCCTCTCC Rv:GATAATCACGTGTGCTGCAGATCCTCCTCAGG
"TEV protease cleavage site" pET-28-TEV-PEX5(ΔN137)	Fw:[Phos]TATGGAGAACCTTTATTTCCAGGGCCA Rv:[Phos]TATGGCCCTGGAAATAAAGGTTCTCCA



	No. of Met in intact protein	No. of Met in smallest PK-resistant species
H_6.PEX5	14	11/10
H_6.PEX5(1-324)	8	5/4
H_6.PEX5(1-197)	6	3/2

FIGURE S1

FIGURE S1. Determination of molar ratios of DTM-bound PEX5 molecules. A, N-terminal sequence of the histidine-tagged PEX5 species used in this work. The conserved cysteine at position 11 is marked, as are all methionine residues (bold, underlined). The cleavage site of Genenase I (Gen. I ✂) and the approximate cleavage site of PK (PK ✂) that yields the smallest PK-protected stage 2 PEX5 species are also shown. Note that there is some uncertainty in defining the latter cleavage site. Thus, two possibilities are considered: PK cleaves after methionine 18 or PK cleaves before methionine 18. The number of methionine residues in both intact PEX5 and smallest PK-protected stage 2 PEX5 species are presented in the table on the right side. **B-E**, Quantitative analyses of autoradiography data. Densitometric analysis of radiolabeled proteins bands was performed with ImageQuant® software (**B**). For each selected gel area (red squares), a single volume value was determined that includes both intact and truncated PEX5 species. Quantitation was performed with a local median background correction. Values for ^{35}S-PEX5(C11A) were multiplied by 3, as only 1/3 of the *in vitro* reactions were loaded onto the gels. To determine the contribution of each individual band, in PEX5(1-197;C11A) and PEX5(1-324;C11A) experiments, intensity profiles along intersecting red lines were plotted (**C**). The intensity profiles (**D**) were then deconvoluted using Fityk software (1) (**E**); the thin grey line represents the raw data obtained with ImageQuant®; the black line represents the assisted fitting of each peak (peaks I to V); the blue lines represent the deconvoluted peaks as determined by Fityk. **F**, molar ratios of DTM-bound truncated PEX5(C11A) species to full-length PEX5(C11A). Signal intensities for each of the resolved PEX5 species (peaks I-V in E) were normalized for the number of methionine residues considering three different possibilities. In the first possibility (scenario #1) we assumed that: 1) the smallest PK-protected PEX5 species (peak I in E) has the same size, and therefore the same number of methionines, of Genenase I-cleaved PEX5 species (see Fig. 4A); 2) the largest PK-cleaved PEX5 species (peak IV in E) is larger than the corresponding untagged PEX5 species (see Fig. 5B); and 3) the difference in size between any two adjacent PK-resistant species in the SDS-gel is approximately 1 kDa (see Fig. 3). In the two other possibilities we just assumed that band/peak IV is larger than the untagged protein (see Fig. 5B) and that all the other PK-accessible species contain either the minimum (scenario #2) or the maximum number of possible methionines (scenario #3). Thus, these two possibilities yield the largest and smallest ratios of truncated PEX5 species to full-length PEX5, respectively. Graphical representation of scenarios #2 and #3 for PEX5(1-197;C11A):PEX5(C11A) and PEX5(1-324;C11A):PEX5(C11A) are presented (see main text for scenario #1). The data were fitted to a dose response one-site specific binding curve, and divided by the Bmax value obtained for full-length PEX5(C11A). Thus, values in the ordinate represent molar ratios of PEX5(1-324;C11A) or PEX5(1-197;C11A) to PEX5(C11A) (averages and standard deviations from four replicates are shown). In scenario #2, the Bmax for PEX5(1-197;C11A) and PEX5(C11A) are 3.24 (95% CI = 2.32-5.87) and 1.00 (95% CI = 0.90-1.11), respectively (left panel); and the Bmax for PEX5(1-324;C11A) and PEX5(C11A) are 1.35 (95% CI = 0.97-2.28) and 1.00 (95% CI = 0.85-1.18), respectively (right panel). In scenario #3, the Bmax for PEX5(1-197;C11A) and PEX5(C11A) are 1.82 (95% CI = 1.31-3.24) and 1.00 (95% CI = 0.90-1.11), respectively (left panel); and the Bmax for PEX5(1-324;C11A) and PEX5(C11A) are 1.02 (95% CI = 0.75-1.69) and 1.00 (95% CI = 0.85-1.18), respectively (right panel). Note that the abscissa scale is different for the two radiolabeled proteins. Note that intact PEX5(1-324;C11A) and intact PEX5(1-197;C11A) data cannot be fitted to the same dose response curve ("ambiguous fit"); in these cases the lines simply connect averages. Note also that for some points the error bars are shorter than the height of the symbols.

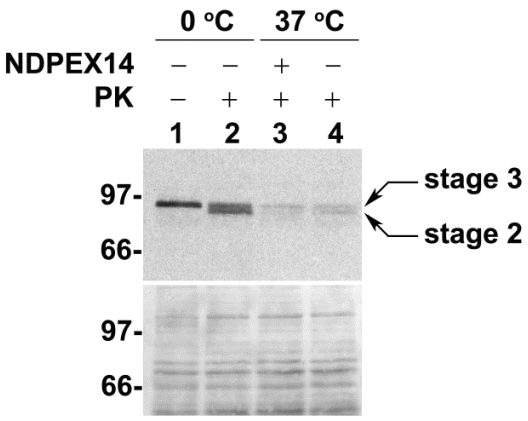
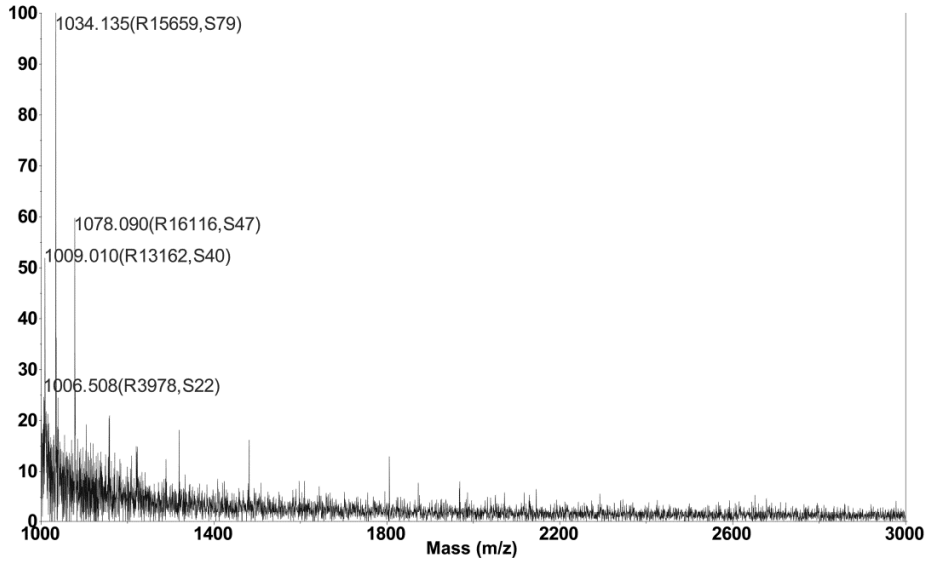


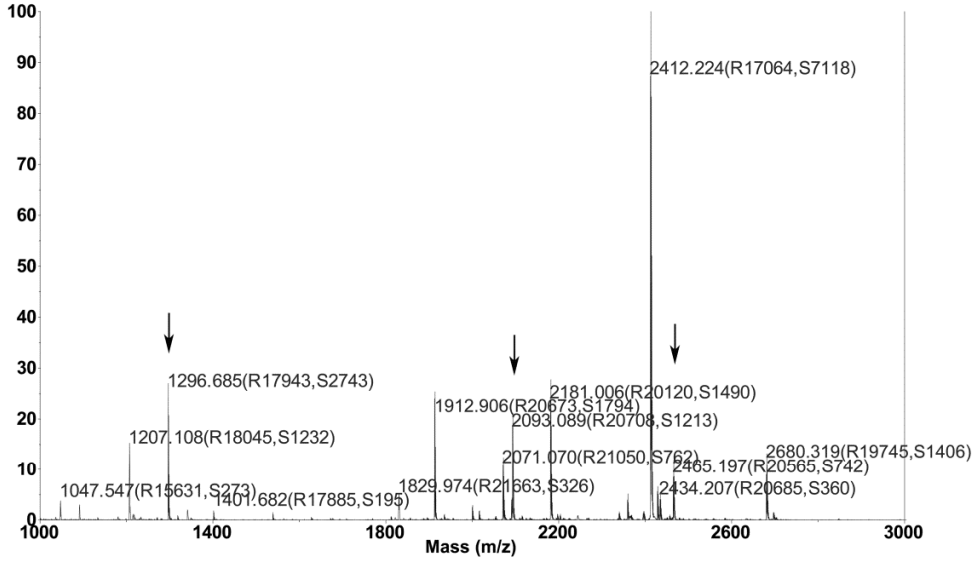
FIGURE S2

FIGURE S2. The amount of organelle-associated PK-protected endogenous PEX5 is largely decreased upon incubation at 37 ºC in the presence of ATP. A rat liver PNS in ATP-containing import buffer was incubated at 0 or 37 ºC in the absence (lanes 1, 2 and 4) or presence (lane 3) of 10 μM recombinant NDPEX14. After incubation, samples were treated (+) or not (–) with PK, as indicated. Organelles were then isolated and subjected to reducing SDS-PAGE/western-blotting. Endogenous rat PEX5 was detected by blot-overlay using ^{35}S-PEX14. Stage 2 and stage 3, DTM-embedded non-ubiquitinated and monoubiquitinated PEX5 species, respectively. Note that the Ub-PEX5 thioester conjugate is destroyed under reducing conditions. Thus, only the full-length PEX5 protein is detected in reducing gels (2). The autoradiograph (upper panel) and the corresponding Ponceau S-stained membrane (lower panel) are shown. Numbers to the left indicate the molecular weight (kDa) of protein standards.

A



B



C

FindPept output.

Experimental mass (Da)	Theoretical mass (Da)	Δmass (ppm)	Peptide	Position
1912.906	1912.902	-2.000	(M)GSSHHHHHHSSGLVPRGS(H)	2-19
2071.070	2071.067	-1.200	(M)AMRELVEAEAGGANPLMKLA(G)	22-41
2181.006	2181.002	-2.000	(M)GSSHHHHHHSSGLVPRGSHM(A)	2-21
2181.006	2181.002	-2.000	MGSSHHHHHHSSGLVPRGSH(M)	1-20
2412.224	2412.216	-3.200	(M)AMRELVEAEAGGANPLMKLAGHF(T)	22-44
2680.319	2680.316	-1.200	(S)HMAMRELVEAEAGGANPLMKLAGHF(T)	20-44

FIGURE S3

FIGURE S3. Mass spectrometry analyses of intact and Genenase I-cleaved recombinant PEX5(1-197;C11A) in the reflectron positive mode. Genenase I cleaves particularly well at sites having a histidine at the P2 position and a phenylalanine or a tyrosine at the P1 position (3). Although there are two such sequences in human PEX5 (His23Phe24 and His578Phe579) previous work suggests that only the first of these sites is cleaved by Genenase I (4). To determine whether Genenase I cleaves PEX5(1-197;C11A) at the first of these sites (corresponding to residues His43Phe44 in the histidine-tagged PEX5(1-197;C11A) protein) the intact and Genenase I-digested proteins were analyzed by mass spectrometry. No peptides were found in the undigested protein (A) whereas five major peptides were detected in the Genenase I-cleaved protein (B). The five peptides map to the 1- 44 amino acid region of recombinant PEX5(1-197;C11A), as determined by the FindPept program (5) using the "unspecific cleavage" option and a mass tolerance of 10 ppm (C). In B, arrows indicate the internal standards added to the sample.

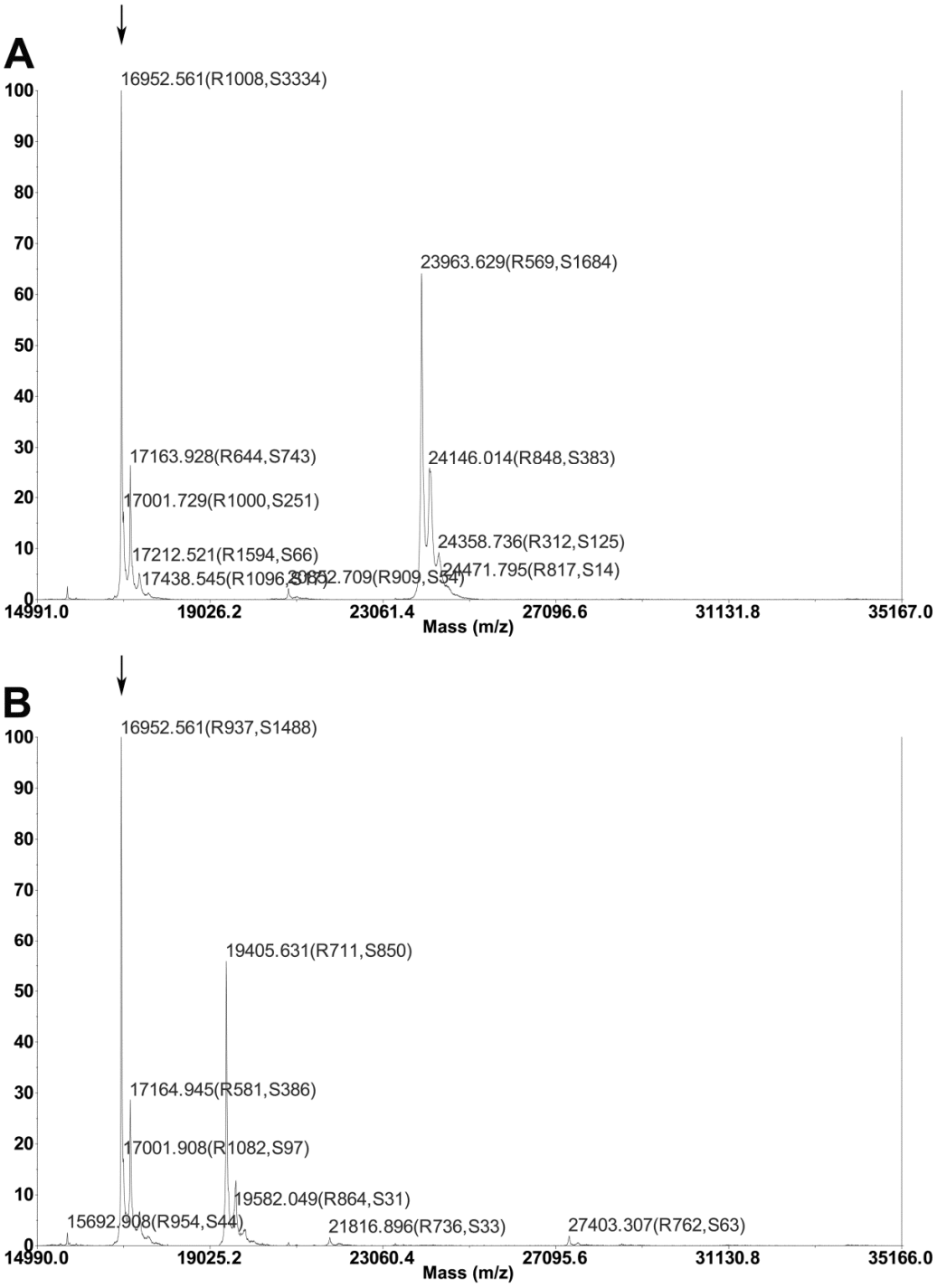

A
16952.561(R1008,S3334)
23963.629(R569,S1684)
17163.928(R644,S743)
24146.014(R848,S383)
17001.729(R1000,S251)
24358.736(R312,S125)
17212.521(R1594,S66)
24471.795(R817,S14)
17438.545(R1096,S85)
20852.709(R909,S54)
100
90
80
70
60
50
40
30
20
10
0
14991.0
19026.2
23061.4
27096.6
31131.8
35167.0
Mass (m/z)
B
16952.561(R937,S1488)
19405.631(R711,S850)
17164.945(R581,S386)
17001.908(R1082,S97)
19582.049(R864,S31)
15692.908(R954,S44)
21816.896(R736,S33)
27403.307(R762,S63)
100
90
80
70
60
50
40
30
20
10
0
14990.0
19025.2
23060.4
27095.6
31130.8
35166.0
Mass (m/z)

FIGURE S4

FIGURE S4. Mass spectrometry analyses of intact and Genenase I-cleaved recombinant PEX5(1-197;C11A) in the linear positive mode. Masses of 23963.6 Da (MH+) for the undigested protein (theoretical mass 23961.172 Da (MH+), excluding the initial methionine) and 19405.6 Da for the Genenase I-cleaved protein were determined. This suggests that Genenase I removes a domain of 4576 Da from recombinant PEX5(1-197;C11A), in good agreement with the theoretical molecular mass of 4576.13 Da for a peptide comprising amino acid residues 2-44 of the protein. In A and B, the arrow indicates the internal mass standard horse myoglobin.

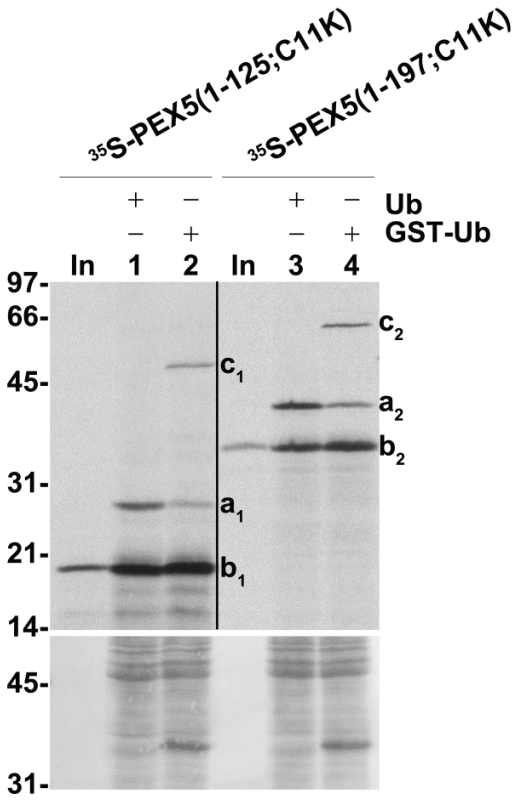


FIGURE S5

FIGURE S5. Control assay showing that the slower migrating radioactive bands detected in PNS-based *in vitro* assays programmed with PEX5(1-125;C11K) and PEX5(1-197;C11K) upon SDS-PAGE represent monoubiquitinated species. Radiolabeled PEX5(1-125;C11K) or PEX5(1-197;C11K) were subjected to PNS-based *in vitro* assays in the presence of AMP-PNP and either ubiquitin (Ub; lanes 1 and 3) or a glutathione S-transferase-ubiquitin fusion protein (GST-Ub; lanes 2 and 4). Organelles were isolated by centrifugation and analyzed by SDS-PAGE/autoradiography. The autoradiograph (upper panel) and a portion of the corresponding Ponceau S-stained membrane (lower panel) are shown. The exposure time of the PEX5(1-125;C11K) panel was 4-fold larger than the one of PEX5(1-197;C11K). Lanes In, RRL containing the radiolabeled proteins, as indicated. a and c, Ub-PEX5 and GST-Ub-PEX5 species, respectively; b, non-ubiquitinated PEX5 species. Note that, in lanes 2 and 4, Ub-PEX5 species containing endogenous ubiquitin are also observed. Numbers to the left indicate the molecular weight (kDa) of protein standards.

REFERENCES

1. Wojdyr, M. (2010) Fityk : a general-purpose peak fitting program. *J. Appl. Crystallogr.* **43**, 1126–1128

2. Carvalho, A. F., Pinto, M. P., Grou, C. P., Alencastre, I. S., Fransen, M., Sá-Miranda, C., and Azevedo, J. E. (2007) Ubiquitination of mammalian Pex5p, the peroxisomal import receptor. *J. Biol. Chem.* **282**, 31267–72

3. Carter, P., Nilsson, B., Burnier, J. P., Burdick, D., and Wells, J. A. (1989) Engineering subtilisin BPN' for site-specific proteolysis. *Proteins*. **6**, 240–8

4. Gouveia, A. M., Guimarães, C. P., Oliveira, M. E., Reguenga, C., Sá-Miranda, C., and Azevedo, J. E. (2003) Characterization of the peroxisomal cycling receptor, Pex5p, using a cell-free in vitro import system. *J. Biol. Chem.* **278**, 226–32

5. Gattiker, A., Bienvenut, W. V., Bairoch, A., and Gasteiger, E. (2002) FindPept, a tool to identify unmatched masses in peptide mass fingerprinting protein identification. *Proteomics*. **2**, 1435–1444