Supporting Information

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SI Methods

All plasmids were constructed using standard protocols (1). Plasmids GFP-UB(G76V), GFP-UB(G76V)ko, and UB(G76V)-GFP were purchased from Addgene. GFP-LC3 was kindly provided by Dr. Yoshinori Ohsumi (National Institute of Basic Biology, Okazaki, Japan). LAMP1-Cherry and LAMP1-GFP were obtained from Dr. George Patterson (National Institutes of Health, Bethesda, MD).

UB-RFP was constructed by excising the ubiquitin ORF (ORF) from UB(G76V)-GFP with *Nhe*I and *Bam*HI and ligating the resulting fragment into similar sites in pmRFP-N1 (2). UBko-RFP was constructed by amplifying the ORF of the lysine ubiquitin mutant from GFP-UB(G76V)ko using the synthetic forward and reverse oligonucleotides fp 5′-CGGAGCTCCACCATGCAGATCTTCGTC-3′ and rp 5′-CTGGATCCCGCACACCTCTTAGTCTTAAG-3′. The resulting products were then digested with *Sac*I and *Bam*HI and ligated into equivalent sites in pmRFP-N1. UBkomut-RFP was constructed using the Quikchange protocol of Stratagene and the following pairs of mutagenic oligonucleotides in three consecutive mutagenesis reactions: 1) I44A fp 5′-CAACAAAGATTGGCCTTTGCCGGTAGA-3′ and rp 5′- TCTACCGGCAAAGGCCAATCTTTGTTG -3′; 2) L8A fp 5′-CTTCGTCAGAACGGCCACCGGTAGAACCATAAC-3′ and rp 5′- GTTATGGTTCTACCGGTGGCCGTTCTGACGAAG -3′; and 3) V70A fp 5′-CGACCTTACATCTTGCCTTAAGACTAAGAGG-3′ and rp 5′- CGACCTTACATCTTGCCTTAAGACTAAGAGG -3′.

The construction of PMP34-GFP, GFP-SKL, and PEX3-GFP has been described previously (2). PMP34-GFP-UB and PMP34-GFP-UBko were constructed by digesting GFP-UB(G76V) and GFP-UB(G76V)ko, respectively, with *Bsr*GI and *Not*I and then ligating the resulting fragments, individually, into similar sites in PMP34-GFP. UB-GFP-SKL was constructed by digesting UB(G76V)-GFP with *Nhe*I and *Bsr*GI and ligating the resulting fragments it to similar sites in GFP-SKL. Similarly, UBko-PEX3-GFP was constructed by excising the PEX3-GFP ORF from PEX3-GFP using *Bgl*II and *Not*I and then ligating it into *Bam*HI-*Not*I-digested UBko-RFP. To construct PEX3-GFP-UBko, the ORF of PEX3-GFP was amplified from PEX3-GFP using the oligonucleotides fp 5′-CGAGATCTCCTGAGGTCTGTATGG-3′ and rp 5′-GATCTAGAGTCGCGGCCGC-3′. The resulting PCR products were then digested with *Bgl*II and *Not*I and ligated into *Bam*HI-*Not*I-digested UBko-RFP.

CFP-LC3 was constructed by digesting mCFP with *Nhe*I and *Bsr*GI and then ligating the resulting fragment into similar sites in GFP-LC3.

1. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning - A Laboratory Manual,* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).
2. Kim PK, Mullen RT, Schumann U, Lippincott-Schwartz J (2006) The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. *J Cell Biol* 173:521–532.

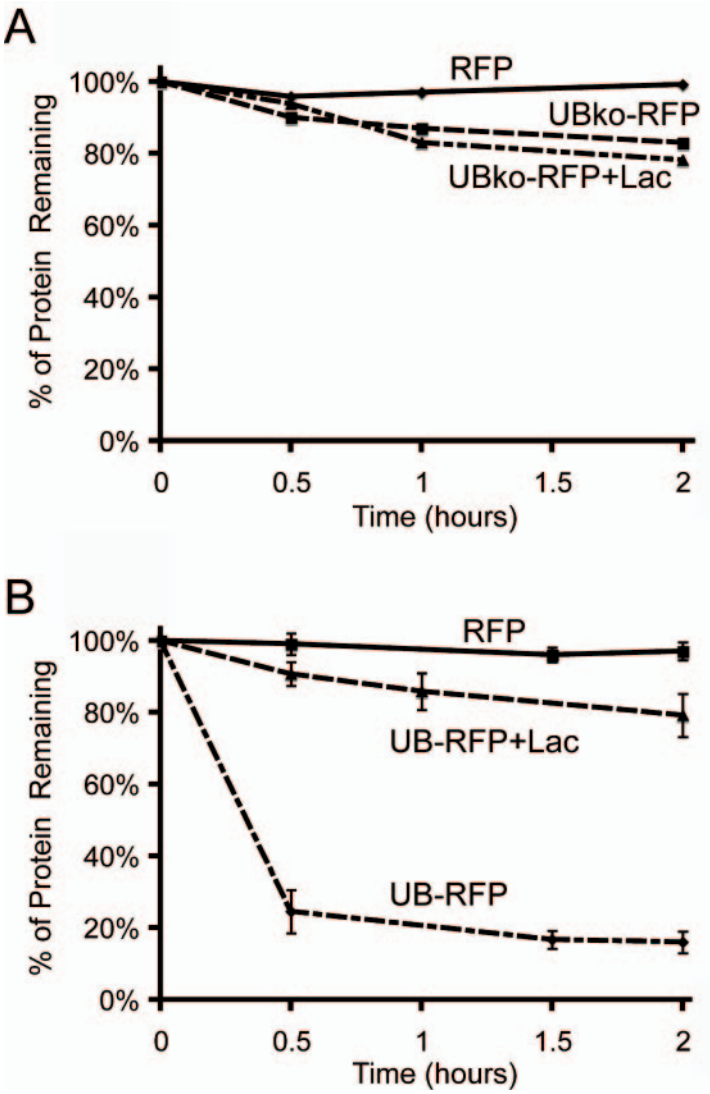

Fig. S1. UB-RFP, but not UBko-RFP, is a substrate of the proteasome. The relative rates of RFP (*A* and *B*), UB-RFP (*A*), and UBko-RFP (*B*) protein degradation were analyzed by [^{35}S] pulse labeling (for 15 min) followed by immunoprecipitations of protein products from lysate of transiently-transfected COS-7 cells over time. Cells expressing UB-RFP or UBko-RFP were also treated with or without 10 μM lactacystin (+Lac) starting 30 min before the pulse reaction. The percent of immunoprecipitated protein remaining at each time point, compared to that at the initiation of the chase, was quantified by densitometry. Shown are the averages $\pm$ standard deviations of three independent experiments. Note that in (*A*) the UB-RFP protein degraded rapidly with a half-life of approximately 15 min. However, when UB-RFP-expressing cells were treated with lactacystin, a proteasome inhibitor, the degradation of UB-RFP was significantly diminished ($P < 0.01$). On the other hand, UBko-RFP was not degraded by the proteasome, since lactacystin treatment did not affect the rate of UBko-RFP protein degradation (*B*). Note also that the rate of UBko-RFP degradation was slightly greater than that of RFP alone (*B*), suggesting that another mechanism(s) is responsible for its degradation.

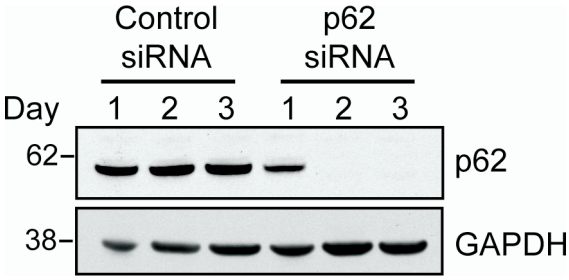

Fig. S2. Silencing of p62 expression using siRNA. HeLa cells were transfected with either control siRNA or p62 siRNA pools over a 24-h period: first at day 0 and second at day 1 (see *Experimental Procedures* for details). Immunoblot of HeLa cells lysate at 1, 2, and 3 days after the first transfection treatment with either control siRNA or p62 siRNA treatment is shown. Day 1 represents cell lysate 24 h after the first transfection but before the second transfection treatment. Days 2 and 3 represent cell lysate s24 and 48 h after the second transfection treatment, respectively. Cells were lysed and 25 μg total protein was subjected to SDS/PAGE and then immunoblotted with antibodies against either p62 or GAPDH serving as a loading control.

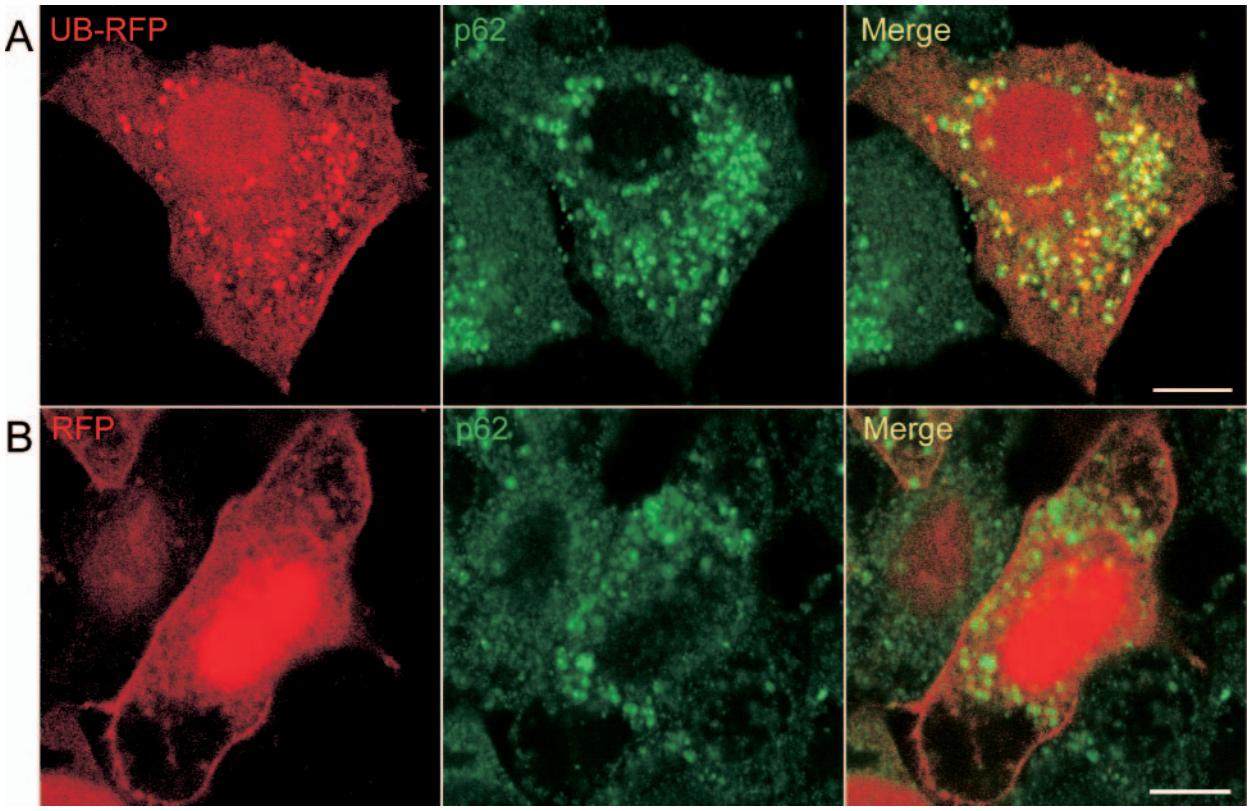

Fig. S3. COS-7 cells transiently expressing either UB-RFP (*A*) or RFP (*B*). Cells were fixed 24 h after transfection and then immunostained with anti-p62 and Alexa 488 goat anti-rabbit antibodies. (Scale bar, 10 μm.)

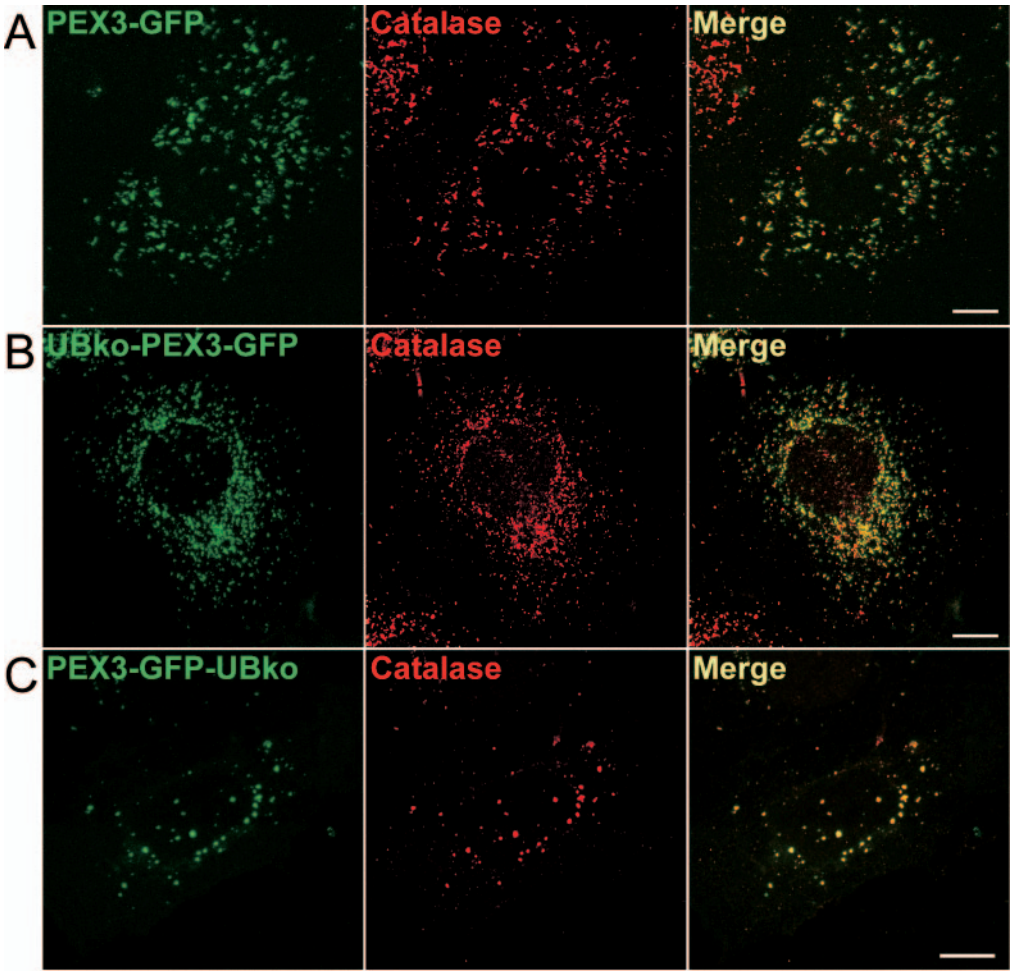

Fig. S4. Ubiquitin must be located on the cytosolic surface of the peroxisomal boundary membrane for peroxisome degradation to occur. COS-7 cells transiently transfected with PEX3-GFP (*A*), UBko-PEX3-GFP (*B*), or PEX3-GFP-UBko (*C*). Cells were fixed 48 h after transfection and then stained with anti-catalase and Alexa 543-goat anti-rabbit antibodies. All images shown are maximum projections of a z-series.