

Appendix

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Figure S1: Analysis of PEX5 in USP30 WT and KO cells.

Figure S2: Analysis of protein ubiquitylation in USP30 WT and KO cell lysates and mitochondria enriched fractions.

Figure Legend Appendix

Appendix Figure S1 –PEX5 is not differentially ubiquitylated in USP30 KO lysates.

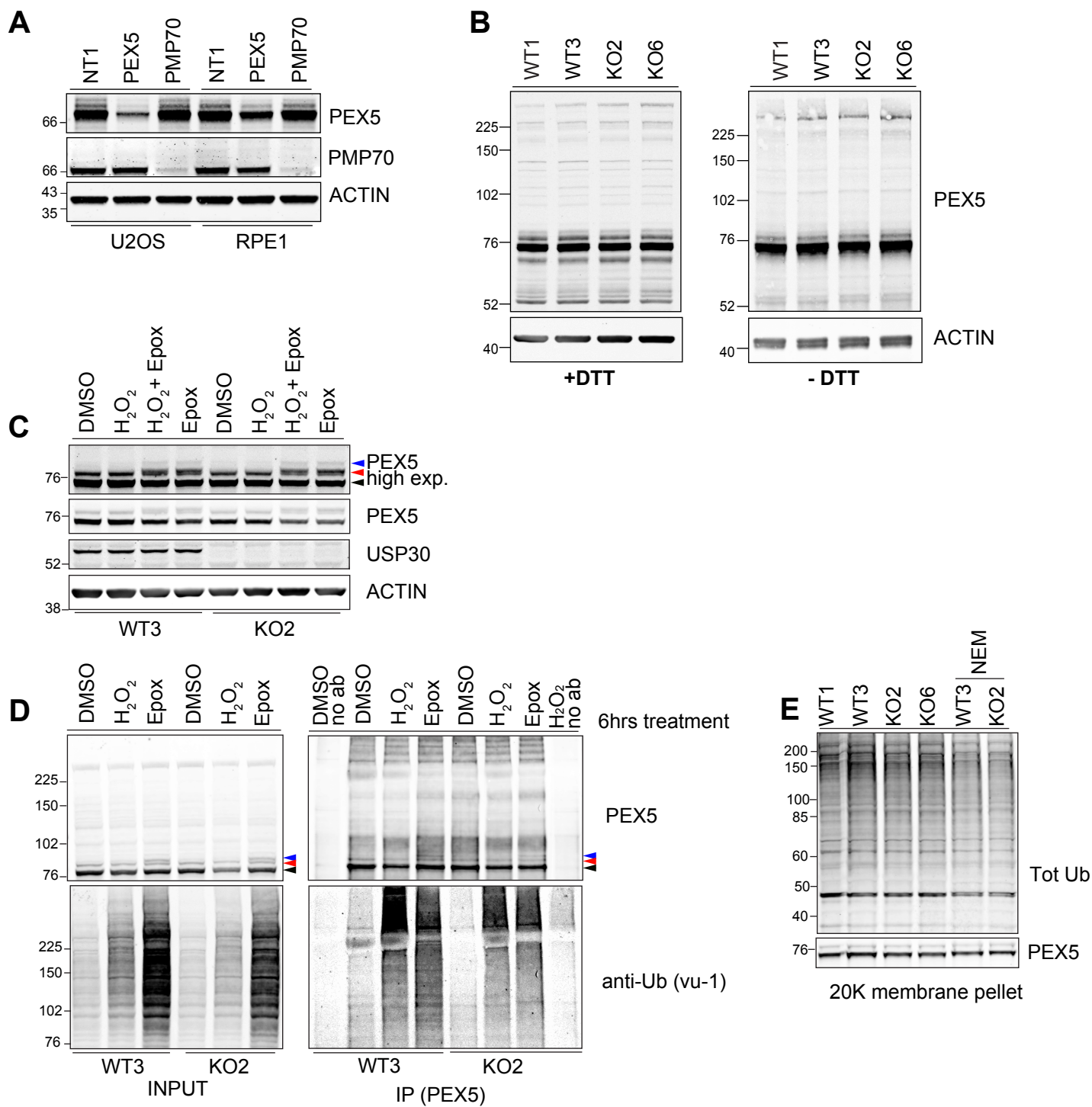
- (A) Validation of PEX5 antibody in U2OS and hTERT-RPE1 cells transfected for 72 h with non-targeting (NT1), PEX5 and PMP70 targeting siRNA.
- (B) Representative western blot of hTERT-RPE1 WT and KO cells (WT1, WT3, KO2 and KO6) analysed under reducing (+DTT) and non-reducing (-DTT) conditions to assess Cys11-ubiquitylation of PEX5.
- (C) Representative western blot of hTERT-RPE1 WT and KO cells (WT3 and KO2) treated for 6 h with 0.4 mM H₂O₂ and/or 100nM Epoxomycin (Epox), lysed in buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100 and protease inhibitors. Black arrow-head indicates full length, red and blue arrowhead, modified PEX5 species.
- (D) Representative western blot of endogenous PEX5 immunoprecipitated from hTERT-RPE1 WT and KO cells (WT3 and KO2) treated for 6 h with 0.4 mM H₂O₂ or 100 nM Epoxomycin (Epox) and lysed in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100 and protease inhibitors. Black arrow-head indicates full length, red and blue arrowhead, modified PEX5 species.
- (E) Representative western blot of 20K membrane pellets (incl. peroxisomes) derived from hTERT-RPE1 WT and KO cells (WT1, WT3, KO2 and KO6). Cells were homogenised in HIM buffer supplemented with fresh proteases inhibitor alone or with 10 mM NEM. A post-nuclear supernatant (PNS) was obtained and first centrifuged for 15 min at 7000 g. The post-mitochondrial supernatant was then centrifuged at 20,000 g for 15 min and the pellet (peroxisomes enriched fraction) was resuspended in SDS-sample buffer and subjected to western blot analysis.

Appendix Figure S2 – Ubiquitylation profile of USP30 WT and KO cell lysates and mitochondria enriched fractions.

- (A) Representative western blot of a TUBE (Life Sensor, UM402) pull down from hTERT-RPE1 WT and KO cells (WT3 and KO2), treated for 6 hours with 100 nM Epoxomycin, then lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 10% Glycerol and fresh protease inhibitors. Equal amount of lysates were incubated with agarose-TUBE beads for 1 h at 4°C. After three washes in TBS including 0.1% Tween, samples were eluted in SDS-sample buffer, incubated 5 min at RT and analysed by western blotting. Membranes were incubated overnight either just with VU1 antibody (total ubiquitin) or first incubated with GFP-K6 affimer (Avacta, 0.1 µg/ml– [1]) prior to incubation with anti-GFP.
- (B) Representative western blot of a TUBE pull down from hTERT-RPE1 WT and KO cells (WT1 and KO2), treated for 2 h with Oligomycin A and Antimycin A (both 1 µM, O/A), then lysed as in (A). Both input (15 µg) and beads were analysed by SDS-PAGE and immunoblotted with TOMM20 antibody.
- (C) Representative western blot of purified mitochondria from hTERT-RPE1 WT and KO cells (WT1 and KO2). Mitochondria were isolated using the commercially available Mitochondria Isolation Kit (Miltenyi Biotec, 130-094-532) following the manufactures' instructions with minor modifications.

1. Michel MA, Swatek KN, Hospenthal MK, Komander D (2017) Ubiquitin Linkage-Specific Affimers Reveal Insights into K6-Linked Ubiquitin Signaling. *Mol Cell* **68**: 233-246 e235

Appendix Figure S1



Appendix Figure S2

