New aspects of USP30 biology in the regulation of pexophagy

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Abstract:

Mitochondria and peroxisomes have a number of features in common: they each play interconnected roles in fatty acid and reactive oxygen species (ROS) metabolism and, once damaged, need to be removed by specialised autophagic mechanisms, termed mitophagy and pexophagy respectively. Both processes can use ubiquitin as an initiating signal but while mitophagy has been extensively studied, pexophagy remains rather poorly understood. Our recent work, along with a new study from Kim and colleagues, has shed light on the molecular mechanism of pexophagy and the importance of reversible ubiquitylation in its regulation. Collectively, both studies highlight the physiological role of the deubiquitylase USP30 in suppressing the turnover of peroxisomes.

Peroxisomes are multifunctional organelles involved in a wide range of metabolic processes within the cell, such as the **α** and β-oxidation of very long chain fatty acids and the synthesis of bile acids and ether phospholipids [1, 2]. Recently more specialised tasks, like the regulation of the antiviral response have highlighted the existence of additional cross-talk between peroxisomes and other organelles within the cell [3-5]. One of the most intriguing relationships is the one peroxisomes share with mitochondria. Mass spectrometry studies have revealed a clear overlap between the proteome of these two organelles. Examples of shared proteins include FIS1 and the GTPase DNM1L/DRP1, which are known to play a role in the fission processes of mitochondria [6, 7] as well as peroxisomes [8, 9]. In recent years, a mitochondrial derived vesicle pathway has been described, whereby selected proteins, exemplified by the ubiquitin E3 ligase MUL1, are packaged into small vesicles derived from the outer membrane of mitochondria and delivered to peroxisomes [10]. Furthermore,

McBride and colleagues have proposed a role for mitochondrial derived vesicles in the biogenesis of newly formed peroxisomes [11].

Together mitochondria and peroxisomes represent two major sources of ROS metabolites within cells and consequently both need to be rapidly eliminated when they are damaged or otherwise non-functional. One important pathway for safe disposal, selective autophagy, commonly uses ubiquitin as a signal to recruit ubiquitin binding domain encoding adapters (for example OPTN/Optineurin, TAX1BP1, SQSTM1/p62 and NBR1), that in turn bind to the phagophore membrane via their LC3-interacting motif (LIR) and allow the engulfment of the damaged organelle into an autophagosome. Fusion of autophagosomes with lysosomes generates autolysosomes in which the degradation process takes place.

Activation of one ubiquitin-dependent mitophagy pathway is now well understood: the kinase PINK1 senses mitochondrial damage, phosphorylates ubiquitin to initiate the pathway, and the E3 ligase PRKN/Parkin amplifies this signal, decorating outer mitochondrial proteins with ubiquitin moieties [12, 13]. The picture is less clear for pexophagy, for which PEX2 has been proposed as the E3 ligase that, in complex with PEX10 and PEX12, ubiquitylates a number of peroxisomal proteins in response to amino acid starvation or ROS [14, 15]. Whilst PINK1 is not implicated in pexophagy (see below), another kinase ATM is recruited to peroxisomes in response to ROS, where it phosphorylates and promotes ubiquitylation of the PEX2-substrate PEX5 [15]. The mechanistic delineation and interpretation of this pathway is however complicated by two facts: firstly, PEX2 is an enzyme that is also engaged in the import of peroxisomal matrix proteins, like the ROS detoxifying enzyme catalase, and thus plays an important role in the generation and maintenance of mature peroxisomes. Secondly, the previously reported acute triggers for pexophagy, starvation and ROS, also induce global autophagy. A specific pexophagy inducing perturbation remains elusive, making its study very challenging.

The mitochondrial deubiquitylase (DUB) USP30 has been shown to oppose depolarisation-induced PINK1-PRKN dependent mitophagy and is rapidly emerging as a potential therapeutic target in Parkinson's Disease [16, 17]. We have recently shown that USP30 also suppresses basal (constitutive) mitophagy (ie in the absence of an acute trigger) in a PINK1-dependent fashion, even in the absence of PRKN. Furthermore, we reported a separate pool of USP30 that is targeted to peroxisomes, where it regulates the selective degradation of

these organelles under basal conditions [18]. Our observations have now been complemented by an independent study that also finds a role for USP30 in starvation induced pexophagy and provides important further insights that warrant a renewed discussion of the potential mechanism and translational avenues (Figure 1) [19].

Immunofluorescence microscopy and cell fractionation data clearly show that a subset of USP30, both tagged (both studies) and endogenous (our study), localises to peroxisomes whilst the bulk of this DUB is targeted to the outer mitochondrial membrane. How then does USP30 reach peroxisomes? USP30 can be targeted to peroxisomes independently of mitochondria, since its reintroduction in cells that were artificially depleted of mitochondria did not prevent its localisation to peroxisomes [18]. We proposed that USP30 may reach peroxisomes through interaction with PEX19, a chaperone that binds to the hydrophobic region of Class I peroxisomal membrane proteins (for example ABCD3/PMP70) to directly target them from free ribosomes to the membrane of mature peroxisomes [20]. In contrast, Riccio et *al.* argue that USP30 can be delivered to new-born peroxisomes from the endoplasmic reticulum (ER), following a previously described PEX16 dependent pathway [21]. Indeed, a chimeric PEX16 construct with an ER-targeting stop-anchor sequence (sa-PEX16) was able to recruit full length USP30 to the ER, revealing another sorting mechanism, that may come into play in the absence of pre-existing peroxisomes.

Given our observation that USP30 is able to modulate basal mitophagy, we reasoned that it may play an equivalent role in basal pexophagy. We made use of a highly sensitive, fluorescent reporter targeted to the peroxisomal matrix (Keima-SKL), that changes its excitation spectrum upon arrival of peroxisomal content in the acidic lumen of the lysosome [22]. Using this pexophagy reporter, we measured an approximately two-fold increase in constitutive pexophagy in cells depleted of USP30 by two independent siRNAs [18]. This increase was dependent on ATG7, confirming the engagement of the canonical autophagy machinery, however it did not require PINK1, in distinction to the USP30-dependent component of basal mitophagy. Moreover, the phenotype could be reproduced in two independent Cas9/CRISPR knockout (KO) cell lines, and both depletion and KO could be rescued by re-introducing wild-type USP30 but not a catalytically-inactive form of the protein. Whilst this two-fold increase in pexophagy was very reproducible, neither the knockdown nor the knockout cell lines showed any evidence of a global decrease in total

peroxisome number (ABCD3 or Catalase positive puncta). We reasoned that the percentage of peroxisomes that are turned over at any one time is most likely too small to impact on any global assessment of peroxisome numbers. Furthermore, we thought it likely that the enhanced pexophagy we observed in our USP30 KO cells was balanced by peroxisome biogenesis.

Riccio et *al.* focused on starvation induced pexophagy, which they assessed by monitoring the reduction in peroxisome density by immunofluorescence (number of ABCD3-positive puncta per cell volume), decrease in peroxisomal protein abundance by western blotting (PEX14) and by using a GFP and RFP tandem-tagged fluorescent pexophagy reporter targeted to the peroxisomal membrane [19]. For each assay, USP30 overexpression was able to rescue the loss of peroxisomes caused by amino acid starvation. Conversely, depletion of USP30 did not affect starvation induced pexophagy, but enhanced the basal turnover of peroxisomes. In agreement with our study, this effect was dependent on the canonical autophagy machinery (ATG12 and ATG5 in this case). The Kim lab previously identified PEX2 as the key E3 ligase that is required for starvation induced pexophagy, and two substrates that are ubiquitylated under these conditions, namely the peroxisomal membrane protein ABCD3 and PEX5 [14]. Co-depletion of PEX2 and USP30 restored peroxisome numbers suggesting that the E3 ligase that USP30 opposes is PEX2. In addition, in cells depleted of USP30, the authors detected ubiquitylated ABCD3 and PEX5, both in basal and in starvation-induced conditions. In our USP30 depleted or KO cells, we failed to find ubiquitylated forms of PEX5 and ABCD3, which may conceivably be too short lived or too minor a species to be detected in our system.

Importantly, Riccio et *al.* reported that USP30 depletion results in a significant loss in ABCD3 positive peroxisomes that was also reflected in lower expression levels of the peroxisomal membrane protein PEX14. This contrasts with our observation that knockdown or knockout of USP30 in a panel of different cell lines did not affect the abundance or the distribution of peroxisomes, neither the expression of peroxisomal membrane nor matrix proteins (further supported by unpublished proteomic datasets). It is unclear at this moment whether this discrepancy is due to different approaches (eg accounting for peroxisomal matrix proteins or not) or cell lines, however any future rationale for the development of USP30 targeted therapies should take note of the potential impact on peroxisomes.

Additional considerations have to be made in the context of certain Peroxisomal Biogenesis Disorders (PBDs), where the expression or activation of USP30 may represent a rational therapeutic strategy. Patient-derived cells bearing the most common mutation in the AAA-ATPase PEX1 (G843D), accumulate ubiquitylated PEX5 on peroxisomes and consequently exhibit increased levels of pexophagy [23]. In this setting, the lysosomotropic agent chloroquine has been shown to restore not only peroxisome number, but also peroxisomal matrix protein import and Very Long Chain Fatty Acid (VLCFA) metabolism, demonstrating a functional rescue of these organelles. Riccio et *al.* now showed that over-expression of USP30 in the same PEX1 (G843D) mutant cells was able to restore the number of ABCD3-positive peroxisomes. One question that needs to be answered is whether these rescued peroxisomes are mature and functional, seeing as they still incorporate a dysfunctional AAA-ATPase.

Collectively, these new data further emphasise the intricate interplay between mitochondria and peroxisomes. Not only is their biogenesis transcriptionally co-regulated but their degradation is also controlled by a single DUB, USP30, which thus plays a central role in limiting the turnover of two major sources of ROS in the cell. Intriguingly, many DUBs that rely on a catalytic cysteine are highly sensitive to inhibition by oxidation [24]. Local inactivation of USP30 by ROS can therefore in principle provide a common and simple mechanism to facilitate disposal of malfunctioning organelles.

In conclusion, these two studies open up a new aspect of USP30 biology and further accentuate its potential as an actionable drug target. Highly selective DUB inhibitors have recently been developed [25-28], however no activators have yet been characterised. Clearly, more work is required to fully dissect the mechanism of basal and induced pexophagy. The discovery of USP30 as a key player in the regulation of this process introduces a new perspective to our understanding of the dynamics of this organelle.

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References:

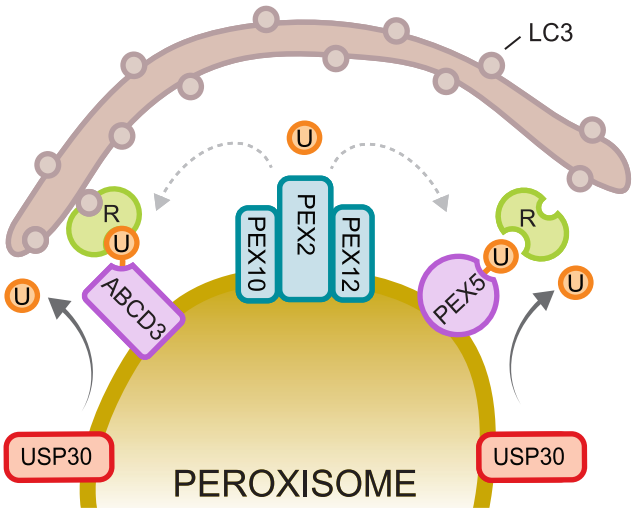
1. Van Veldhoven PP (2010) Biochemistry and genetics of inherited disorders of peroxisomal fatty acid metabolism. *J Lipid Res* **51:** 2863-2895
2. Cipolla CM, Lodhi IJ (2017) Peroxisomal Dysfunction in Age-Related Diseases. *Trends Endocrinol Metab* **28:** 297-308
3. Dixit E *et al* (2010) Peroxisomes are signaling platforms for antiviral innate immunity. *Cell* **141:** 668-681
4. Di Cara F, Sheshachalam A, Braverman NE, Rachubinski RA, Simmonds AJ (2017) Peroxisome-Mediated Metabolism Is Required for Immune Response to Microbial Infection. *Immunity* **47:** 93-106.e107
5. Fransen M, Lismont C (2019) Redox Signaling from and to Peroxisomes: Progress, Challenges, and Prospects. *Antioxid Redox Signal* **30:** 95-112
6. James DI, Parone PA, Mattenberger Y, Martinou JC (2003) hFis1, a novel component of the mammalian mitochondrial fission machinery. *J Biol Chem* **278:** 36373-36379
7. Smirnova E, Griparic L, Shurland DL, van der Bliek AM (2001) Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Mol Biol Cell* **12:** 2245-2256
8. Koch A, Thiemann M, Grabenbauer M, Yoon Y, McNiven MA, Schrader M (2003) Dynamin-like protein 1 is involved in peroxisomal fission. *J Biol Chem* **278:** 8597-8605
9. Koch A, Yoon Y, Bonekamp NA, McNiven MA, Schrader M (2005) A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. *Mol Biol Cell* **16:** 5077-5086
10. Neuspiel M, Schauss AC, Braschi E, Zunino R, Rippstein P, Rachubinski RA, Andrade-Navarro MA, McBride HM (2008) Cargo-selected transport from the mitochondria to peroxisomes is mediated by vesicular carriers. *Curr Biol* **18:** 102-108
11. Sugiura A, Mattie S, Prudent J, McBride HM (2017) Newly born peroxisomes are a hybrid of mitochondrial and ER-derived pre-peroxisomes. *Nature* **542:** 251-254
12. Harper JW, Ordureau A, Heo JM (2018) Building and decoding ubiquitin chains for mitophagy. *Nat Rev Mol Cell Biol* **19:** 93-108
13. Pickrell AM, Youle RJ (2015) The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. *Neuron* **85:** 257-273
14. Sargent G, van Zutphen T, Shatseva T, Zhang L, Di Giovanni V, Bandsma R, Kim PK (2016) PEX2 is the E3 ubiquitin ligase required for pexophagy during starvation. *J Cell Biol* **214:** 677-690
15. Zhang J *et al* (2015) ATM functions at the peroxisome to induce pexophagy in response to ROS. *Nat Cell Biol* **17:** 1259-1269
16. Bingol B, Tea JS, Phu L, Reichelt M, Bakalarski CE, Song Q, Foreman O, Kirkpatrick DS, Sheng M (2014) The mitochondrial deubiquitinase USP30 opposes parkin-mediated mitophagy. *Nature* **510:** 370-375
17. Liang JR, Martinez A, Lane JD, Mayor U, Clague MJ, Urbé S (2015) USP30 deubiquitylates mitochondrial Parkin substrates and restricts apoptotic cell death. *EMBO Rep* **16:** 618-627
18. Marcassa E, Kallinos A, Jardine J, Rusilowicz-Jones EV, Martinez A, Kuehl S, Islinger M, Clague MJ, Urbe S (2018) Dual role of USP30 in controlling basal pexophagy and mitophagy. *EMBO Rep* **19:** e45595
19. Riccio V, Demers N, Hua R, Vissa M, Cheng DT, Strilchuk AW, Wang Y, McQuibban GA, Kim PK (2019) Deubiquitinating enzyme USP30 maintains basal peroxisome abundance by regulating pexophagy. *J Cell Biol*, 10.1083/jcb.201804172

20. Costello JL *et al* (2017) Predicting the targeting of tail-anchored proteins to subcellular compartments in mammalian cells. *J Cell Sci* **130:** 1675-1687
21. 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
22. Katayama H, Kogure T, Mizushima N, Yoshimori T, Miyawaki A (2011) A sensitive and quantitative technique for detecting autophagic events based on lysosomal delivery. *Chem Biol* **18:** 1042-1052
23. Law KB, Bronte-Tinkew D, Di Pietro E, Snowden A, Jones RO, Moser A, Brumell JH, Braverman N, Kim PK (2017) The peroxisomal AAA ATPase complex prevents pexophagy and development of peroxisome biogenesis disorders. *Autophagy* **13:** 868-884
24. Clague MJ (2013) Oxidation controls the DUB step. *Nature* **497:** 49
25. Turnbull AP *et al* (2017) Molecular basis of USP7 inhibition by selective small-molecule inhibitors. *Nature* **550:** 481-486
26. Kategaya L *et al* (2017) USP7 small-molecule inhibitors interfere with ubiquitin binding. *Nature* **550:** 534-538
27. Lamberto I *et al* (2017) Structure-Guided Development of a Potent and Selective Non-covalent Active-Site Inhibitor of USP7. *Cell Chem Biol* **24:** 1490-1500.e1411
28. Gavory G *et al* (2018) Discovery and characterization of highly potent and selective allosteric USP7 inhibitors. *Nat Chem Biol* **14:** 118-125

Figure legend

Figure 1: Proposed model for USP30-dependent regulation of pexophagy.

A discrete pool of USP30 is localised to peroxisomes where it deubiquitylates the peroxisomal membrane protein ABCD3 and the PTS1-receptor PEX5, opposing the ubiquitin E3 ligase PEX2. Ubiquitylated species of ABCD3 and PEX5 serve as a platform for the recruitment of selective autophagy adaptor proteins (p62 and NBR1), that are then recognised and bound by LC3-positive autophagic membranes. (U: Ubiquitin, A: Adaptor).



Marcassa, Figure 1