ATM functions at the peroxisome to induce pexophagy in response to ROS

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Peroxisomes are highly metabolic, autonomously replicating organelles that generate reactive oxygen species (ROS) as a by-product of fatty acid -oxidation. Consequently, cells must maintain peroxisome homeostasis, or risk pathologies associated with too few peroxisomes, such as peroxisome biogenesis disorders, or too many peroxisomes, inducing oxidative damage and promoting diseases such as cancer. We report that the PEX5 peroxisome import receptor binds ataxia-telangiectasia mutated (ATM) and localizes this kinase to the peroxisome. In response to ROS, ATM signalling activates ULK1 and inhibits mTORC1 to induce autophagy. Specificity for autophagy of peroxisomes (pexophagy) is provided by ATM phosphorylation of PEX5 at Ser 141, which promotes PEX5 monoubiquitylation at Lys 209, and recognition of ubiquitylated PEX5 by the autophagy adaptor protein p62, directing the autophagosome to peroxisomes to induce pexophagy. These data reveal an important new role for ATM in metabolism as a sensor of ROS that regulates pexophagy.

Peroxisomes participate in -oxidation of branched and very long chain fatty acids, which results in the production of ROS (refs 1,2). When in excess, ROS can cause cellular damage, and trigger catabolic functions such as autophagy3-6. As autonomously replicating organelles, maintaining the balance between peroxisome biogenesis and degradation is critical for normal cellular homeostasis7-11, and if dysregulated, can give rise to diseases such as peroxisome biogenesis disorders (PBDs)7,11,12, white matter disease13 and Alzheimer’s disease8,13. Although the importance of maintaining peroxisome homeostasis is clear, mechanisms for recognition and removal of excessive or aberrant peroxisomes to prevent pathologies associated with too few or too many peroxisomes are not well understood.

Selective autophagy of peroxisomes (pexophagy) is a major pathway by which excess peroxisomes are eliminated14-18. During selective autophagy, adaptor proteins mediate target recognition, such as the ubiquitin-binding protein p62, which contains both an LC3-interacting region (LIR) that binds to LC3 associated with the nascent autophagosome, and a ubiquitin-associated (UBA) domain that binds to monoubiquitylated lysine residues in the target19. p62 is known to be involved in pexophagy20; however, the peroxisomal targets recognized by p62, and mechanisms responsible for regulation of pexophagy have not been elucidated.

Recently, we reported that ATM signals to the tuberous sclerosis complex (TSC) in the cytoplasm to regulate autophagy in response to ROS (ref. 3). ATM is activated by ROS through formation of a disulphide-crosslinked dimer21, and this kinase has been localized previously to the peroxisome22,23. Importantly, we recently found that the TSC signalling node that regulates mTORC1 (a suppressor of autophagy) is also resident at the peroxisome in liver cells, the predominant cell type in the body for -oxidation of fatty acids24,25. These data led us to reason that ROS may serve as a rheostat for peroxisomal homeostasis, activating signalling molecules at the peroxisome to regulate pexophagy.

RESULTS

ATM is a peroxisome-localized kinase activated by ROS

Endogenous ATM was detected in the nuclear fraction of cells (Fig. 1a), consistent with what is known about the function of this kinase as a DNA damage response sensor26,27. ATM was also found in the membrane and peroxisome compartments (Fig. 1a),
consistent with previous reports that ATM was localized to this organelle\textsuperscript{22,23}. To determine whether peroxisomal ATM localized to the exterior (membrane) or interior (matrix) of this organelle, isolated peroxisomes were treated with proteinase K in the absence or presence of the membrane-disrupting detergent Triton X-100. Like the peroxisome membrane protein PMP70, but not the peroxisome matrix protein catalase that is resistant to degradation when peroxisome membranes are intact, ATM was rapidly degraded in both the absence and presence of Triton X-100, indicating that ATM was associated with the outer (proteinase K accessible) surface of peroxisomes (Fig. 1b).

We also observed an increase in activated ATM in the peroxisome fraction (increased immunoreactivity with a phospho-specific ATM (Ser 1981) antibody) in response to \( \text{H}_2\text{O}_2 \) (Fig. 1c), which was confirmed by deconvolution microscopy, showing co-localization of pATM with the peroxisomal protein catalase in peroxisomes (Fig. 1d). Co-localization was not observed in peroxisome-deficient human fibroblasts from the well-characterized Zellweger peroxisome biogenesis disorder (mutated in the PEX6 gene; Fig. 1d) whereas nuclear localization and activation (phosphorylation) of ATM (pATM) was observed in control and Zellweger fibroblasts (Fig. 1d and Supplementary Fig. 1a). Together, these data identify the peroxisome as a site for activation of ATM in response to ROS.

**ATM is localized to the peroxisome by PEX5**

Peroxisomal proteins are targeted to this organelle by peroxisome import receptors, such as PEX5 (ref. \textsuperscript{28}). ATM was co-immunoprecipitated with PEX5, and activated ATM (pATM) binding to PEX5 was increased by \( \text{H}_2\text{O}_2 \) (Fig. 2a). ATM has been reported to contain a putative PEX5-binding sequence (SRL) at its carboxy terminus\textsuperscript{23} (Fig. 2b). We introduced an arginine (R) to glutamine (Q) mutation into wild-type (WT) ATM at amino acid 3047 (R3047Q) (RQ-ATM) of the SRL (Fig. 2b). RQ-ATM localization to the peroxisome fraction of cells was greatly decreased (Fig. 2c), as was binding to PEX5 (Fig. 2d). Furthermore, WT-ATM showed a cytoplasmic and punctate co-localization with the peroxisome membrane protein PMP70, which increased in \( \text{H}_2\text{O}_2 \)-treated cells, whereas RQ-ATM remained primarily nuclear, and exhibited little co-localization with PMP70 (Fig. 2e,f). However, the intrinsic ability of this ATM mutant to be activated by ROS, and recognize DNA damage was not compromised. ATM is oxidized into an active dimer in response to \( \text{H}_2\text{O}_2 \) (ref. \textsuperscript{21}). Both WT-ATM and the peroxisome-localization-deficient RQ-ATM could be activated by \( \text{H}_2\text{O}_2 \) (Supplementary Fig. 1b) and in vitro kinase assays demonstrated that both WT-ATM and RQ-ATM were activated in the presence of the MRN complex and DNA (Supplementary Fig. 1c) and \( \text{H}_2\text{O}_2 \) (Supplementary Fig. 1d). To determine whether...
peroxisome-localization-deficient ATM could still respond to DNA damage, ATM-deficient cells (AT5, GM05849 and HepG2+ATM siRNA) were reconstituted with either WT-ATM or RQ-ATM. ATM-deficient cells (AT5, GM05849) formed a reduced number of γ-H2AX foci immediately after ionizing irradiation, and contained a higher number of residual foci post irradiation. This phenotype was rescued by RQ-ATM, which exhibited a γ-H2AX foci response equivalent to WT-ATM (Supplementary Fig. 1e,f); similar results were obtained using residual chromosome damage as an endpoint (Supplementary Fig. 1g,h). Therefore, loss of peroxisomal localization did not affect the intrinsic kinase activity or ability of ATM to respond to DNA damage.

Peroxosomal ROS activates ATM to repress mTORC1 and induce autophagy

A role for ATM in peroxisome homeostasis would predict that this kinase would be activated by peroxisomal, as well as exogenous ROS. To test this prediction, we next examined whether endogenous ROS produced at the peroxisome could activate ATM signalling. Rat liver FAO cells were treated with clofibrate, which activates PPAR (Supplementary Fig. 2a–c) and induces peroxisomal ROS, as shown by dihydroethidium staining (Fig. 3a), and quantification with DCFDA (Fig. 3b). Clofibrate activated ATM at the peroxisome (Fig. 3c) and repressed mTORC1 signalling (Fig. 3d,e). Clofibrate also caused mTORC1 repression and induction of autophagy in GFP–LC3 MCF7 (Supplementary Fig. 2d,e) and HepG2 (Supplementary Fig. 2f) cells. Importantly, this response was abrogated in the absence of ATM (Fig. 3f) or TSC2 (TSC2+/− mouse embryonic fibroblasts (MEFs); Supplementary Fig. 2g), demonstrating a requirement for both ATM and the TSC signalling node for mTORC1 repression by ROS, as previously shown25.

ULK1, a kinase essential for autophagy in mammalian cells, is activated by AMPK (phosphorylation at Ser 317) and inactivated by mTORC1 (Ser 757) phosphorylation, respectively, and induction of autophagy by peroxisomal ROS. Concomitantly, p62 decreased and LC3-II increased (Fig. 3g), which was blocked by bafilomycin A1 (Supplementary Fig. 2h). Clofibrate also increased formation of autophagosomes visualized as GFP–LC3 puncta (Supplementary Fig. 2e) and by electron microscopy (Supplementary Fig. 3a–f). Together, these data demonstrate that peroxisomal ROS activates ATM signalling to repress mTORC1 and induce autophagy.

ATM phosphorylates PEX5 at Ser 141 in response to ROS

The minimal essential requirement for the substrates of ATM kinase is S*T+Q, and hydrophobic amino acids at N − 1 and
Although both WT-ATM and the RQ-ATM mutant retained kinase in response to ROS (Fig. 4d) and was dependent on ATM (Fig. 4e). The S141A-PEX5 mutant was not recognized by this antibody (Fig. 4c). The increase in PEX5 immunoreactivity to this antibody in response to ROS. ATM (Supplementary Fig. 4c), confirming the ATM-dependence of phosphorylation motif (Supplementary Fig. 4b). PEX5 was recognized of other ATM substrates, such as p53, matches this consensus ATM phosphorylation motif in PEX5 at Ser 141 (Supplementary Fig. 9). Source data for a can be found in Supplementary Table 1.

Ser 141 regulates PEX5 ubiquitylation at Lys 209 in response to ROS

Ubiquitin on the peroxisome membrane surface is thought to target peroxisomes for degradation, and membrane-bound PEX5 can be ubiquitylated, although the regulation and specific site(s) of ubiquitylation in peroxisomes targeted for pexophagy have not been elucidated. We observed markedly increased PEX5 ubiquitylation when ATM was activated by ROS (Fig. 5a). As PEX5 is known to be both mono- and polyubiquitylated, we examined PEX5 ubiquitylation in response to ROS using HA–Ub–WT and HA–Ub–K0 (ubiquitin with all lysine residues mutated to arginine to prevent chain formation and polyubiquitylation) co-expressed with Myc–PEX5. No decrease in the apparent molecular weight, nor amount of PEX5 ubiquitylation in response to ROS using the HA–Ub–K0 construct (that can only be monoubiquitylated), compared with the HA–Ub–WT construct (Fig. 5b), was observed, indicating that PEX5 was monoubiquitylated in response to ROS.

Figure 3 Peroxisomal ROS activates ATM to repress mTORC1 and induce autophagy. (a) Representative images of FAO cells treated with vehicle (dimethylsulphoxide) or 0.25 mM clofibrate for 1 h; superoxide production was detected using dihydroethidium (DHE) staining. Scale bars, 30 μm. (b) Representative data of the DCFDA assay from two independent experiments depicting the levels of ROS in FAO cells treated with clofibrate at the indicated doses for 1 h. Tert-butyl hydroperoxide (TBHP) 50 μM was used as a positive control for the assay. (c) ATM activation was monitored at the peroxisome in response to clofibrate at 3 h (1 mM) as indicated by western blot analysis of whole-cell extract (WCE) and peroxisomal fractions (P) obtained from FAO cells. (d,e) FAO cells treated with 1 mM clofibrate for the indicated time points; activation of ATM–AMPK–TSC2 signalling and suppression of mTORC1 was monitored by western analysis for pATM (Ser 1981), ATM, pS6K (Thr 389), S6K, pS6 (Ser 235/236), S6, p4EBP1 (Thr 37/46), 4EBP1, AMPK, ACC, pULK1 (Ser 757, mTORC1 site), pULK1 (Ser 317, AMPK site) and ULK1. (f) Western analysis of HEK293 cells transfected with control or ATM siRNA and treated with 0.5 mM clofibrate for 6 h using anti-pS6K (Thr 389), S6K, pS6 (Ser 235/236), S6, p4E-BP1 (Thr 37/46) and 4E-BP1 antibodies. (g) FAO cells treated with clofibrate (1 mM) for the indicated times. Induction of autophagy was monitored by western analysis of p62 and LC3-II. Unprocessed original scans of blots are shown in Supplementary Fig. 9. Source data for b can be found in Supplementary Table 1.

N – 3; negatively charged amino acids at N + 1 are positive determinants for substrates phosphorylated by ATM (ref. 30). Kinase prediction (http://scansite.mit.edu) identified a potential ATM S'\(S/T\)Q phosphorylation motif in PEX5 at Ser 141 (Supplementary Fig. 4a,b). This highly conserved sequence in PEX5, similar to that of other ATM substrates, such as p53, matches this consensus ATM phosphorylation motif (Supplementary Fig. 4b). PEX5 was recognized by a pan-phospho-(S/T) ATM substrate antibody; immunoreactivity significantly increased with \(H_2O_2\) (Fig. 4a), was blocked by the ATM inhibitor KU55933 (Fig. 4b), and was lost in AT fibroblasts lacking ATM (Supplementary Fig. 4c), confirming the ATM-dependence of increased PEX5 immunoreactivity to this antibody in response to ROS.

Site-directed mutagenesis of Ser 141 of PEX5 to alanine (S141A) revealed that immunoreactivity with the pan-phospho-(S/T) ATM substrate antibody increased with \(H_2O_2\) treatment in WT-PEX5, but the S141A-PEX5 mutant was not recognized by this antibody (Fig. 4c). A polyclonal antibody generated against the phospho-Ser-141-PEX5 peptide confirmed that phosphorylation of PEX5 at Ser 141 increased in response to ROS (Fig. 4d) and was dependent on ATM (Fig. 4e). Although both WT-ATM and the RQ-ATM mutant retained kinase activity (see above), the RQ-ATM mutant failed to phosphorylate PEX5 at Ser 141 (Fig. 4f). Together, these data demonstrate that PEX5 is phosphorylated on Ser 141 in response to ROS, and this phosphorylation is ATM-dependent.
ATM phosphorylates PEX5 at Ser 141 in response to ROS. (a) Immunoprecipitation of lysates from HEK293 cells overexpressing Myc–PEX5 and treated with H2O2 (0.4 mM) for 1 h, using an anti-Myc antibody followed by immunoblotting with a phospho-(S/T) ATM substrate antibody. Inputs were immunoblotted using the indicated antibodies. (b) Immunoprecipitation performed with anti-Myc antibody of lysates from HEK293 cells overexpressing Myc–PEX5, treated with H2O2 (0.4 mM) for 1 h in the presence/absence of an ATM inhibitor (KU-55933, 2 h pretreatment) followed by immunoblotting with phospho-(S/T) ATM substrate antibody. Inputs were immunoblotted using the indicated antibodies. (c) HEK293 cells transfected with either a Myc–PEX5-WT or Myc–PEX5-S141A mutant construct treated with H2O2 (0.4 mM) for the indicated times. Immunoprecipitation was performed with an anti-Myc antibody followed by immunoblotting with phospho-(S/T) ATM substrate antibody. Inputs were immunoblotted using the indicated antibodies. (d) HEK293 cells transfected with either a Myc–PEX5-WT or Myc–PEX5-S141A mutant construct treated with H2O2 (0.4 mM) for 1 h. Western analysis was performed with anti-pPEX5 (Ser 141), Myc, pATM (Ser 1981), ATM and GAPDH antibodies. (e) HEK293 cells transfected with Myc–PEX5-WT for 24 h following a siRNA knockdown of ATM for 48 h treated with H2O2 (0.4 mM) for 1 h. Western analysis was performed with anti-pPEX5 (Ser 141), Myc, pATM (Ser 1981), ATM and GAPDH antibodies. (f) AT (GM05849) fibroblast cells were transfected with Myc–Pex5 and Flag–ATM-WT or Flag–ATM-RQ mutant for 48 h and treated with H2O2 (0.4 mM) for 1 h. Western analysis was performed with anti-pPEX5 (Ser 141), Myc, pATM (Ser 1981), ATM and GAPDH antibodies. Unprocessed original scans of blots are shown in Supplementary Fig. 9.

Figure 4

Prediction of ubiquitylation sites on PEX5 using PhosphoSite (http://www.phosphosite.org) and a series of constructs generated by site-directed mutagenesis were used to identify Lys 209 as a major site for PEX5 ubiquitylation (Fig. 5c,d and Supplementary Fig. 5a). A search of mass spectrometry databases [http://www.phosphosite.org] revealed that Lys 209 of PEX5 had been identified by mass spectrometry as being ubiquitylated, confirming Lys 209 as a bonafide binding site for PEX5 ubiquitylation.

Phosphorylation can modulate protein ubiquitylation by regulating E3 ligase recognition or subcellular localization of substrates, leading us to ask whether phosphorylation of PEX5 by ATM regulated PEX5 ubiquitylation in response to ROS. Mutation of the Ser 141 ATM phosphorylation site in PEX5 abrogated increased ubiquitylation of PEX5 in response to ROS (Fig. 5e), indicating that phosphorylation at this site regulated ROS-induced PEX5 ubiquitylation. In agreement with these data, ROS-induced ubiquitylation of endogenous PEX5 was deficient in ATM-null cells (Supplementary Fig. 5b) and following ATM knockdown with short interfering RNA (siRNA; Supplementary Fig. 5c). Low background PEX5 ubiquitylation was seen with both WT- and S141A-PEX5 (Fig. 5e), suggesting that there may be other sites of ubiquitylation that are not dependent on ATM phosphorylation at Ser 141, nor ROS-responsive.

The RING peroxins PEX2, PEX10 and PEX12 are part of a peroxisome-localized E3 ligase responsible for polyubiquitylation of PEX5 (ref. 34), and as expected, siRNA knockdown of these peroxins reduced polyubiquitylation of PEX5 (Supplementary Fig. 5d). Knockdown of these E3 ligases also reduced monoubiquitylation of PEX5, suggesting this peroxisomal E3 ligase also participates in PEX5 monoubiquitylation (Supplementary Fig. 5e). Collectively, these data indicate that PEX5 is ubiquitylated in response to ROS, and that this ubiquitylation is regulated by ATM phosphorylation.

Ubiquitylated PEX5 binds to p62 to target peroxisomes for pexophagy

We next examined whether the autophagy adaptor p62, which contains both LIR (autophagosome) and UBA (ubiquitin) binding domains, recognizes ubiquitylated PEX5 to mediate pexophagy. The binding of endogenous PEX5 to p62 was enhanced by ROS (Fig. 6a),
and although most of the PEX5 import receptor is cytosolic, co-localization of PEX5 in peroxisomes with p62 and ubiquitin was increased by H₂O₂ (Fig. 6b). Whereas total p62 decreased in response to H₂O₂, p62 association with peroxisomes increased markedly in response to ROS (Fig. 6c). p62 binding to peroxisomes was confirmed by co-localization of p62 with the peroxisome marker PMP70 in liver cells (Fig. 6d). Moreover, co-localization of PMP70 with FIP200 (ULK1 complex component) or ATG16L1 (ATG5 complex component), both essential for autophagosome formation, significantly increased with H₂O₂ (Supplementary Fig. 6a).

Ubiquitylation of PEX5 to tether p62 to the peroxisome was further supported by decreased K209R-PEX5 in the peroxisome fraction (Fig. 6e) and decreased p62 binding to K209R- versus WT-PEX5 (Fig. 6f). Similarly, WT-PEX5 binding to p62 was increased by ROS (Supplementary Fig. 6b) and decreased with the K209R-PEX5 mutant in HEK293 cells (Supplementary Fig. 6c). However, the K209R mutation decreased basal PEX5 binding to p62, and attenuated the increase in p62 binding to PEX5 observed in response to ROS (Fig. 6f), although some p62 binding to K209R-PEX5 could be detected, albeit at much lower levels, suggesting that other sites of ubiquitylation on PEX5 may also be recognized by this autophagy adaptor, perhaps in response to other stimuli. Together, these data show that the autophagy adaptor protein p62 binds to PEX5, and that ubiquitylation at Lys 209 by ROS increases this interaction.

**Induction of pexophagy by ROS**

Pexophagy is a naturally occurring process for regulation of peroxisome number, which can also lead to selective destruction of peroxisomes in response to metabolic signals. In cells stably co-expressing GFP–LC3 (an autophagosome marker) and DsRed–SKL (a peroxisome marker), autophagosomes were visualized to sequentially tether and engulf peroxisomes in response to ROS (Supplementary Fig. 7a and Supplementary Video 1), with a concomitant decrease in peroxisomal proteins PEX1 and PEX14 (Fig. 7a,b). Bafilomycin A1 blocked this decrease, and markedly increased pPEX5 (Ser 141) in the peroxisome fraction, confirming that ROS had increased autophagic flux/pexophagy (Supplementary Fig. 7b–d). Interestingly and in contrast, little mitophagy was seen in response to ROS (Fig. 7a,b). ROS-induced pexophagy was also demonstrated by using peroxisome-localized EGFP–SKL and mRFP–SKL (ref. 41). In response to ROS, co-localization of the relatively stable mRFP signal with the more labile EGFP signal decreased as peroxisomes engulfed by autophagosomes fused with lysosomes in cells undergoing pexophagy (Fig. 7c,d and Supplementary Video 2). Increased pexophagy in response to ROS was further confirmed by electron microscopy (Supplementary Figs 3a–f and 8a–d, respectively); no decrease of PEX1 and PEX14 protein levels was observed in response to ROS in p62 knockdown cells (Supplementary Fig. 7e,f).

A requirement for ATM signalling to induce pexophagy and maintain peroxisome homeostasis would predict that cells that were
ATM- or TSC2-deficient (downstream effector for suppression of mTORC1) would have elevated levels of ROS due to an inability to maintain peroxisome homeostasis. We and others have found that both ATM-deficient human lymphoblasts from patients with ataxia-telangiectasia (AT) disease and TSC2-deficient MEFs have elevated levels of ROS relative to wild-type cells. Following knockdown of PEX5, reconstitution of cells with either WT-PEX5, S141A- (ATM-phosphorylation deficient), S141E- (phosphomimetic) or K209R- (ROS-induced ubiquitylation-deficient) PEX5 mutants suggested that pexophagy (decreased PEX1 and PEX14) was attenuated in cells expressing S141A or K209R PEX5 mutants, but not the S141E phosphomimetic PEX5 mutant (Fig. 7e,f). However, the S141E mutation did not induce pexophagy, indicating that phosphorylation (and ubiquitylation) was not sufficient to induce pexophagy (Fig. 7e,f).

Pexophagy (decreased PEX14-positive puncta, PEX1 and PEX14 protein level, and co-localization of EGFP and mRFP) was observed in response to ROS in ATM-proficient cells, but not ATM-deficient AT cells (Fig. 7g,h,i and Supplementary Fig. 7g,h). Similarly, ROS-induced pexophagy was observed in ATM-deficient cells reconstituted with WT-ATM, but not kinase-dead ATM (Supplementary Fig. 7i), and was abrogated by knockdown of the PEX2/10/12 E3 ligase (Supplementary Fig. 7j), confirming the key role played by ATM in signalling pexophagy in response to ROS.

**DISCUSSION**

ATM-mediated suppression of mTORC1 and phosphorylation of PEX5 by ROS provides an attractive mechanism whereby ROS acts as a rheostat to induce pexophagy to prevent oxidative damage to the cell from excessive or defective peroxisomes. As shown in the model in Fig. 8, we demonstrated that in addition to suppressing the autophagy inhibitor mTORC1 (ref. 3), the ATM kinase triggers pexophagy by inducing PEX5 phosphorylation and subsequent ubiquitylation, resulting in p62 binding and targeting of peroxisomes for degradation.

Selective autophagy has been observed for many cellular organelles. During selective autophagy, autophagy receptors play a key role in targeting of the autophagosome to organelle. In mammals, the most
Figure 7 Induction of pexophagy by ROS. (a) HepG2 cells treated with either H$_2$O$_2$ (0.4 mM) for the indicated times or CCCP (50 μM, 6 h). Western analysis of the peroxisome proteins PEX1 and PEX14 (SDHA and VDAC as mitochondrial markers, p62 and LC3-II as autophagy markers). (b) Quantification of PEX1 and PEX14 intensity normalized to GAPDH from a (mean ± s.d., n=3 independent experiments, Student’s t-test). *P < 0.05 and ***P < 0.001; NS, not significant. (c) Representative images using HepG2 cells transfected with an mRFP–EGFP–SKL construct and treated with H$_2$O$_2$ for 6 h. Scale bars, 10 μm. High-magnification images of the outlined areas are indicated to the right (scale bars, 2.5 μm). (d) Pearson’s correlation coefficient for co-localization between mRFP–SKL and EGFP–SKL was calculated from c. n = 4 independent experiments; 10 cells were analysed in each experiment. All error bars represent s.d., (Student’s t-test) ***P < 0.001. (e) HEK293 cells transfected with Myc–PEX5-WT or Myc–PEX5-K209R or Myc–PEX5-S141A or Myc–PEX5-S141E for 24 h following prior siRNA knockdown of PEX5 for 48 h, treated with H$_2$O$_2$ (0.4 mM) for 6 h. Western analysis was performed using anti-PEX1, PEX14, phosphor-ATM (Ser 1981), ATM, Myc and GAPDH antibodies. Corresponding immunoblots for HEK293 cells transfected with control or PEX5 siRNA showing levels of PEX5. (f) Quantification of PEX1 and PEX14 intensity normalized to GAPDH from e. (Mean ± s.d., n=4 independent experiments, Student’s t-test.) ***P < 0.001; NS, not significant. (g) WT (GM08399) and AT (GM05849) fibroblasts were treated with H$_2$O$_2$ (0.4 mM) for 6 h and immunoblotted with PEX1, PEX14, phospho-ATM, ATM and GAPDH antibodies. (h) Representative images using WT (GM08399) and AT (GM05849) fibroblasts transfected with an mRFP–EGFP–SKL construct and treated with H$_2$O$_2$ for 6 h. Scale bars, 15 μm. (i) Pearson’s correlation coefficient for co-localization between mRFP–SKL and EGFP–SKL was calculated from h. Quantification was performed from Supplementary Fig. 6a on n=3 independent experiments; 10 cells were analysed in each experiment. All error bars represent s.d., (Student’s t-test) – NS, not significant. Unprocessed original scans of blots are shown in Supplementary Fig. 9. Statistic source data for b,d,f and i can be found in Supplementary Table 1.

We show that one mechanism for inducing selective autophagy of peroxisomes is activation of ATM, phosphorylation and ubiquitylation of PEX5, and binding of the autophagy adaptor p62. Interestingly, in Pichia pastoris, PEX5 has been shown to be a redox-regulated protein, where H$_2$O$_2$ decreases import of PTS1 proteins into peroxisomes. PEX14 has also been reported to bind LC3-II under conditions of amino acid starvation and overexpression of the peroxisomal membrane protein PMP34, fused with a ubiquitin on the cytosolic face of peroxisomes, is sufficient to trigger turnover of peroxisomes.

prevalent autophagy-targeting signal is the modification of cargo by ubiquitylation. For example, selective autophagy of damaged mitochondria (mitophagy) occurs after the mitochondrial kinase PINK1 phosphorylates the mitochondrial outer membrane protein mitofusin 2, inducing its ubiquitylation by recruiting the E3 ligase parkin, or directly phosphorylating the E3 ligase parkin to trigger mitophagy. However, for other organelles, including the peroxisome, details of the molecular mechanism(s) underlying cargo recognition and trafficking during selective autophagy have been lacking.
Figure 8 Working model for peroxisomal ATM signalling to the TSC signalling node to repress mTORC1, phosphorylation of PEX5 to induce ubiquitylation by PEX2/10/12 E3 ligase, and recognition of Ub–PEX5 by p62 to induce pexophagy in response to ROS.

Whether these peroxisomal proteins are also targets of the ATM kinase, or regulated by other, yet to be identified mechanisms, is unknown. There is also evidence that in addition to p62, the autophagy adaptor NBR1 can also participate in mammalian pexophagy, suggesting that other pathways in addition to p62 binding to PEX5 for selectively targeting peroxisomes for autophagy may also exist.

PEX5 as a target for the ATM kinase is particularly attractive. PEX5 is known to be ubiquitylated after docking at the peroxisome membrane, becoming either polyubiquitylated and targeted for proteasome-mediated degradation, or monoubiquitylated for recycling back to the cytosol. Our data reveal a previously unknown role for PEX5 as a target of the ATM kinase, which when phosphorylated at Ser 141 becomes ubiquitylated at Lys 209 and serves as a target for the autophagy adaptor p62, providing yet another role (pexophagy) for ubiquitylation of PEX5 at the peroxisome.

Our data show that ATM signalling at the peroxisome participates in pexophagy through two pathways. The first is activation of AMPK and TSC2, leading to repression of mTORC1. mTORC1 is a well-known inhibitor of autophagy, and relief of this repression through AMPK activation and phosphorylation of ULK1 at Ser 317 would increase autophagic flux. The second is phosphorylation of PEX5, triggering ubiquitylation of this peroxisomal protein, and binding of the autophagy adaptor protein, p62, targeting peroxisomes for pexophagy. Data showing that the phosphomimetic S141E-PEX5 mutation alone was unable to induce pexophagy in the absence of ATM activation by ROS suggest that both mTORC1 repression and PEX5 phosphorylation are important, and phosphorylation (and ubiquitylation) of PEX5 may be necessary, but not sufficient, to induce pexophagy.

So far, studies on the role of cell signalling in peroxisome homeostasis have primarily focused on the role of cell signalling pathways in peroxisome biogenesis through regulation of transcription of genes required for peroxisome biogenesis. For example, drugs such as hypolipidemic fibrates that act as PPARα ligands transcriptionally upregulate genes that promote peroxisome biogenesis. Importantly, in response to PPARα activation, genes for peroxisome-localized metabolic processes that generate ROS are disproportionately upregulated relative to those for ROS scavengers, resulting in increased ROS generation. The resultant oxidative stress is thought to contribute to the hepatocarcinogenicity of PPARα ligands in rodents. Reactive intermediates generated at the peroxisome include free radicals such as superoxide and H2O2, and reactive nitrogen species (RNS). Many free radical scavengers, including catalase and superoxide dismutase, are specifically targeted to the peroxisome to protect the cell from peroxisomal ROS and RNS. Interestingly, we have recently shown that ATM can also be activated by RNS to repress mTORC1 to induce autophagy. This suggests the interesting possibility that ATM may also be activated by RNS produced by peroxisomes, and that both ROS and RNS could act as rheostats for cellular sensing of excessive or aberrantly functioning peroxisomes and induction of pexophagy to maintain homeostasis. As ROS can be produced by other organelles, it will be interesting to determine whether ROS produced at other sites activates ATM and induces pexophagy, or whether mechanisms exist to prevent peroxisomes from being targeted for pexophagy in response to ROS produced elsewhere in the cell. There are several possible mechanisms by which cells could regulate pexophagy in response to ROS to provide organelle-specificity. For example, when oxidized by ROS, ATM forms an active dimer. We do not know at this time whether PEX5 recognizes and binds ATM as a monomer or a dimer. Whether ROS produced by other organelles can lead to pexophagy and/or ATM-mediated phosphorylation/ubiquitylation of PEX5 at the...
sites we identified (Ser 141 and Lys 209) is also not known. Although Ser 141 seems to be necessary for ROS-induced ubiquitylation, we do not know whether there are other sites/modifications that occur on PEX5 (or other peroxisomal proteins) that contribute to specificity, modifications that could occur only at peroxisomes, or specifically in response to peroxisomal ROS/RNS. These interesting hypotheses now await further testing.

Although the role of ATM in the DNA damage response in the nucleus is well known, cytoplasmic functions for ATM are now emerging. Interestingly, an ATM-R3047X mutation with a truncated C terminus is well known, cytoplasmic functions for ATM are now emerging. Modifications that could occur only at peroxisomes, or specifically in PEX5 (or other peroxisomal proteins) that contribute to specificity, not know whether there are other sites/modifications that occur on Ser 141 seems to be necessary for ROS-induced ubiquitylation, we do.

### METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

### ACKNOWLEDGEMENTS

We thank S. Subramani (University of California, San Diego, California) for critical advice and providing the mRF-EGFP-SKL plasmids, and M. Mancini (Baylor College of Medicine, Houston, Texas) for providing the DsRed–SKL plasmid. We are also grateful for the assistance of K. Dunne (UTMD. Cancer Center, Houston, Texas) and D. Townley (Baylor College of Medicine, Houston, Texas) in electron microscopy image acquisition. This work was supported by National Institutes of Health (NIH) Grant R01 CA148113 to C.L.W., a Robert A. Welch Endowed Chair in Chemistry (BE-0023) to C.L.W., and NIH grants CA129537, CA154320 and GM109768 to T.K.P. J.J. was a recipient of the China Scholarship Council (CSC).

### AUTHOR CONTRIBUTIONS

J.Z., D.N.T., J.J. and C.L.W. designed research; J.Z., D.N.T., J.J., K.A., A.A., R.T.P., R.D., J.T.-M., J.-H.L., R.K.P. and V.K.C. performed research; J.Z., D.N.T., J.J., R.D., T.T.P., T.K.P., M.B.K. and C.L.W. analysed data; J.Z., D.N.T. and C.L.W. wrote the manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/rcb3230

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Flag–ATM-WT (no. 31985) and HA–p62 (no. 28027) plasmids were purchased from Addgene. Flag–ATM-R3047Q mutant constructs were generated by the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies). R3047Q: FW 5'-GACCCCAAAAATCTCAGCCAACTTTTCCCCAGGTGGAAGC-3'; RV 5'-GCTTTCCAATCTGGGGTCATC-3'.

HA–Ub (no. 18712) and HA–Ub-K0 (no. 17603) plasmids were purchased from Addgene. DiRed–PTS1 was provided by M. Mancini (Baylor College of Medicine, USA), pAT-003 mRFP-EGFP-SKL was provided by S. Subramani (University of California, San Diego, USA). Specificity of mutagenesis was confirmed by direct sequencing using the Sequencing and Microarray Core at U.T.M.D. Anderson Cancer Center, Houston, Texas.

**RNAi knockdown.** On-TARGETplus AT mRNA (L-003201-00-005), SiRNA against human PEX2, PE10X and PE12x mRNA (M-006548-02, M-006545-00, and M-019375-02), siSMARTpool human PEX5 (M-015788-00) and siSMARTpool human SQSTM1 (M-010230-00) were purchased from Dharmacon. siRNAs were resuspended in 1X siRNA buffer (GE Dharmacon) to obtain a 20 μM stock. HEK293 cells were transfected with the indicated siRNA at 10 nM final concentrations with DharmaFECT 1 (GE Dharmacon) according to the manufacturer's instructions for 72 h before collection of lysates.

**Cellular fractionation.** Cytosolic, nuclear and membrane fractions were obtained as described previously. Peroxisomes were isolated using the Peroxisome Isolation Kit (Sigma) as the manufacturer's instructions. Briefly, cells (~2 x 10⁶) were homogenized in 1 x peroxisome extraction buffer, and centrifuged at 1,000 g for 10 min. The supernatant was centrifuged at 2,000 g for 10 min, and collected and further centrifuged at 25,000 g for 20 min. The pellet was resuspended in 400 μl 1 x peroxisome extraction buffer, and subjected to density gradient centrifugation at 100,000 g for 90 min. The peroxisome-enriched fraction was collected from the bottom layer of the density gradient.

**Immunoprecipitation and western blotting assay.** The cells were washed in PBS 3 times and lysed directly using CST lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na pyrophosphate, 1 mM β-glycerophosphate for 30 min at 4°C. The lysis buffer contained 1 x protease inhibitor cocktail and phosphatase inhibitor cocktail 2 and 3 (Sigma)). Lysates were microcentrifuged at 30°C at 10,000 g for 10 min. The supernatant was subjected to BCA Protein Assay (Thermo Scientific) to quantify protein levels. For immunoprecipitation, the cell lysates were incubated with the indicated antibodies and Magnetic A/G beads (Thermo Scientific) overnight at 4°C. The beads were washed and dried with lysis buffer 5 times and were subjected to 5 min denaturing loading buffer for 10 min at 95°C before being resolved by SDS-PAGE. The cell lysates were separated on a 4–15% gel (Bio-Rad), transferred to PVDF membranes and probed with antibodies. Densitometric analysis for quantification of expression levels was performed using ImageQuant software and data were normalized with GAPDH expression. Student's t-test (two-tailed) was performed on at least three biological repeats using GraphPad Prism software. Error bars represent standard deviations of normalized fold changes.

**Protease protection assay.** The crude peroxisomal fraction was isolated using the Peroxisome Isolation Kit (Sigma). The fractionation sample was separated into two groups: Group 1, Proteinase K (Roche) 0.1 μg/ml; Group 2, Proteinase K 0.1 μg/ml and 1% Triton X-100. Both groups were incubated on ice for 5, 15 and 30 min respectively. Phenylmethylsulfonyl fluoride was used to stop the reaction and samples were processed by western blot assay.

**γ-H2AX foci analysis.** Cells were cultured in chamber slides followed by fixation, and immunostaining for detection of phosphorylated H2AX as previously described. Fluorescent images of foci from 100 cells for each experiment were captured as described previously. Nuclear sections were captured, and images obtained by projection of the individual sections as described previously.

**Chromosomes aberrations analysis.** Ionizing-radiation-induced chromosomal aberrations were analyzed at metaphase. Cells in exponential phase were irradiated with 3 Gy, incubated for 9 h post irradiation, treated with colcemid for 3 h and then fixed stained with Giemsa. Metaphase chromosomes were analysed as described previously. Categories of asymmetric chromosome and chromatid aberrations scored included dicentrics, centric rings, interstitial deletions, acentric rings, breaks and gaps, exchanges. For each experiment, fifty metaphases were analysed and each experiment was repeated three times.
ATM kinase assay. Kinase assays were performed in kinase buffer (50 mM HEPES, pH 7.5, 50 mM potassium chloride, 5 mM magnesium chloride, 10% glycerol, 1 mM ATP and 1 mM dithiothreitol) for 90 min at 30 °C in a volume of 40 μl. Kinase assays with oxidation were performed in the absence of dithiothreitol with 817 μM H₂O₂, 0.4 mM or 0.8 mM ATM, 100 nM GSTp53 substrate.

Electron microscopy. The cells were plated on chamber slides for electron microscopy, and treated with vehicle or clofibrate or H₂O₂. The samples were fixed using 2% glutaraldehyde in 100 mM sodium cacodylate with 2 mM CaCl₂ at room temperature. Images were obtained at the High Resolution Electron Microscopy Facility at U.T.M.D. Anderson Cancer Center and Baylor College of Medicine.

Immunofluorescence microscopy. Cells were plated on coverslips and maintained at 37 °C and 5% CO₂ for 24 h before staining. Cells were washed with 1 x phosphate-buffered saline (PBS) three times and fixed in 4% paraformaldehyde for 15 min, permeabilized in 0.5% Triton X-100 for 10 min, blocked with 3.75% BSA in PBS for 1 h at room temperature, and incubated with primary antibody overnight at 4 °C. Secondary antibodies were applied for 1 h at 37 °C, stained with DAPI for 2 min and mounted using SlowFade Gold Antifade reagent (Life Technologies). Images were captured using either a Deltavision Deconvolution Microscope (DeltaVision Elite, GE) or a Nikon confocal system. Live cell imaging was performed using a Deltavision Deconvolution Microscope equipped with a sCMOS camera, and a temperature-controlled CO₂ incubation chamber. Images were acquired with a x60/1.42 oil objective (Olympus). SoftWoRx software was used for acquisition of image stacks, time-lapse and deconvolution. For time-lapse, the cells were plated on glass-bottom microwell dishes (MatTek) for 24 h ahead of time and then immediately treated with H₂O₂ or before image acquisition on the stage. The images were acquired every 3 min with z-stacks at 37 °C and 5% CO₂. The video of stacked images was acquired every 3 min. Images were quantified using ImageJ software. For co-localization analysis, Pearson’s correlation coefficient was calculated using Imaris software V7.6.1 (Bitplane AG). The numbers of PEX14-positive vesicles were calculated using ImageJ. At least 100 cells per condition in 4 independent experiments were used for quantification.

ROS measurement by DCFDA assay and dihydroethidium (DHE) staining. FAO cells were plated in 96-well plates (black bottom) for 24 h and maintained at 37 °C and 5% CO₂. Cells were treated with (0.25 mM, 0.5 mM and 1 mM) clofibrate (Sigma) or dimethylsulphoxide (vehicle control) for 1 h. Tert-butyl hydroperoxide (TBHP) served as a positive control in this experiment. Cells were stained with DCFDA for 30 min, followed by measurement of the absorbance using a fluorescent plate reader (Synergy H1 Hybrid, BioTek) with excitation wavelength at 485 nm and emission wavelength at 535 nm. For DHE staining, FAO cells were plated on chamber slides for 24 h and maintained at 37 °C and 5% CO₂. Cells were treated with 0.25 mM clofibrate (Sigma) for 1 h or dimethylsulphoxide (vehicle control). The cells were incubated with 5 μM DHE (in PBS) for 30 min at 37 °C in a dark chamber, fixed for 10 minutes in 4% paraformaldehyde and images were promptly captured using an Olympus BX40 fluorescence microscope.

RNA extraction and quantitative RT–PCR. RNA was extracted using the RiboPure Kit (Life technologies). Briefly, the procedure is as follows: cells were plated in 6-well plates and cultured at 37 °C and 5% CO₂ for 24 h. The cells were washed with PBS three times before scraping in 1 ml TRI Reagent solution (Ambion). One milliliter of the homogenate was transferred to a 1.5 ml centrifuge tube. Two hundred microlitres of chloroform was added and vortexed at maximum speed. Following a 5 min incubation at room temperature, the samples were centrifuged at maximum speed for 10 min. Four hundred microlitres of the aqueous phase was transferred to a new tube followed by addition of 200 μl 100% ethanol. The sample was transferred to a Filter Cartridge-Collection and centrifuged 1 min at maximum speed. The column was washed with 70% ethanol and 100 μl of elution buffer was added to elute RNA. We used the Taq Man Fast Universal Kit for quantitative RT–PCR assay on an ABI Via7. The primers were purchased from Life Technologies and human GAPDH was used as a normal control.

Statistical analysis. The data are shown as mean ± s.d. Student’s t-test (two-tailed) was performed for the comparisons between two groups and P < 0.05 was considered statistically significant. All experiments were repeated at least three times and representative data are shown as indicated.

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Supplementary Figure 1 Response of ATM RQ mutant to oxidative and DNA damage. (a) Subcellular fractionation of Zellweger (GM13267) fibroblasts demonstrating the localization of phospho-ATM (S1981) and ATM in response to H$_2$O$_2$ (0.4 mM, 1 h) in the indicated subcellular compartments. LDH, Lamin A/C and β-integrin were used as markers for cytosol (C), nuclear (N) and membrane (M) fractions, respectively. WCE, whole cell extract. (b) AT (GM05849) fibroblasts were transfected with either wild type (WT) or peroxisome localization-deficient R3047Q (RQ) mutant Flag-tagged ATM and treated with H$_2$O$_2$ (0.4 mM) for 1 h. ATM was immunoprecipitated using anti-Flag antibody and activation determined using phosphoserine S1981 specific antibody. (c and d) ATM kinase assay with MR complex, Nbs1, and DNA were performed with 0.2 nM dimeric ATM, 50 nM GST-p53 substrate, 96 nM MR, 195 mM Nbs1, and 140 nM linear double stranded DNA. Phosphorylation of ATM was detected by SDS-PAGE and western blotting with an antibody to p53 phospho-Ser15. (e) Frequency of β-H2AX foci in AT cells (AT5, GM05849) and HepG2 cells +siATM at different time points post irradiation of 2 Gy. 100 cells were analyzed for each time point in each experiment (Mean ±s.d., n = 3 independent experiments, student’s t test). The mean number of foci is plotted against time. * P < 0.05, ** P < 0.01. (f) Representative figures of γ-H2AX foci. A: Untreated; B: AT5+ATM cells 30 min post irradiation; C: AT5 cells after 30 min of post radiation; D: AT5 cells 180 min post irradiation; E: AT5+ATM cells 180 min post irradiation; F: AT5+ATM-R3047Q cells 180 min post irradiation. Scale bar, 10 μm (g) Representative images of chromosome aberrations at metaphase. A: AT5+ATM control metaphase; B: AT5+ATM showing dicentric (big arrow) and breaks (small arrow); C: AT5 showing radials (big arrow) and breaks (small arrow); D: AT5+ATM-R3047Q showing radials (big arrow) and breaks (small arrow) (h) Chromosome aberrations at metaphase post irradiation of 3 Gy in AT cells (AT5, GM05849) and HepG2 cells +siATM. For each group 50 metaphases were scored in each experiment (mean ±s.d., n = 3 independent experiments, student’s t test). ** P < 0.01. Uncropped images of western blots are shown in Supplementary Fig. S9. Statistical source data for Supplementary Fig. S1e,h can be found in Supplementary Table 1.
Supplementary Figure 2 Peroxisomal activation of ATM signaling to AMPK-TSC2 to repress mTORC1 and induce autophagy. (a) FAO cells treated with clofibrate (0.25 mM) for 48 h activated PPARα to increase the mRNA level of EHHADH by RT-PCR analysis. The data shown is representative of two independent experiments. (b) FAO cells treated with clofibrate (0.25 mM) for 48 h activated PPARα to increase the mRNA level of ACAA1 and EHHADH by RT-PCR analysis. The data shown is representative of two independent experiments. (c) Western analysis of FAO cells treated with clofibrate at 0.25 mM for indicated times with ACAA1 and EHHADH antibodies. (d) MCF7 cells stably transfected with GFP-LC3 were treated with clofibrate (0.25 mM) for indicated times, and activation of ATM-AMPK-TSC2 signaling and induction of autophagy monitored by western analysis using pATM (S1981), ATM, pAMPK (T172), AMPK, pACC (S79), ACC, pS6K (T389), S6K, pS6 (S235/236), S6, p4E-BP1 (T37/46), 4E-BP1, pULK1 (S757), pULK1 (S317), ULK1, p62 and LC3. (e) Representative images of GFP-LC3 puncta from MCF7 cells stably expressing GFP-LC3 and treated with clofibrate (0.25 mM) for 6 h. Scale bar, 10 μm. (f) HepG2 cells treated with 1 mM clofibrate for indicated times, were monitored for ATM-AMPK-TSC2 signaling, suppression of mTORC1, and induction of autophagy by western analysis using pATM (S1981), ATM, pAMPK (T172), AMPK, pACC (S79), ACC, pS6K (T389), S6K, pS6 (S235/236), S6, p4E-BP1 (T37/46), 4E-BP1, pULK1 (S757), pULK1 (S317), ULK1, p62 and LC3. (g) TSC2+/+ and TSC2−/− MEF cells were treated with clofibrate for 6 h at the indicated doses, and suppression of mTORC1 monitored by western analysis using pS6 (235/236), and S6. (h) Western analysis of FAO cells pre-incubated in the presence or absence of Bafilomycin A1 (Baf A1, 200 nM) for 1 h before treatment with 1 mM clofibrate for 3 h using anti-p62 and LC3-II antibodies. Uncropped images of western blots are shown in Supplementary Fig. S9, statistic source data for Supplementary Fig. S2a,b can be found in Supplementary Table 1.
Supplementary Figure S3 Pexophagy in FAO cells with clofibrate. Representative electron microscopy images of FAO cells treated with vehicle or clofibrate. (a) vehicle 6 h, (b) clofibrate 6 h (1 mM) (c) vehicle 24 h, (d) clofibrate 24 h (0.25 mM), (e) clofibrate 48 h (0.25 mM), and (f) clofibrate 72 h (0.25 mM). Scale bar, 0.5 μm. Autophagosomes containing peroxisomes are represented by yellow arrows and shown enlarged in the boxed area (Scale bar, 0.2 μm).
**Supplementary Figure S4** PEX5 contains ATM S*/T*Q phosphorylation motif. (a) Prediction of PEX5 phosphorylation site and ubiquination site by ScanSite and PhosphoSite. (b) Conserved S141 (ATM phosphorylation site) sequence in PEX5. (c) WT fibroblasts (GM08399) and AT fibroblasts (GM05849) were transfected with Myc-PEX5-WT and treated with H₂O₂ for 1 h. The complex was immunoprecipitated with anti-Myc and immunoblotted with phospho-(S/T) ATM substrate antibody. Inputs were immunoblotted using the indicated antibodies. Uncropped images of western blots are shown in Supplementary Fig. S9.
Supplementary Figure S5 ROS-induced PEX5 ubiquitination is ATM-dependent. (a) HEK293 cells expressing Myc-PEX5-WT or PEX5 mutants (K28R, K52R, K170R, K204R, K209R, K292R) and HA-Ub constructs were co-immunoprecipitated using anti-HA and blotting with an anti-Myc antibody. Input lysates were immunoblotted using the indicated antibodies. (b) WT (GM08399) and AT (GM05849) fibroblasts were treated with H₂O₂ for 6 h and immunoprecipitated with anti-PEX5 and immunoblotted with anti-Ub antibodies. Input lysates were immunoblotted using the indicated antibodies. (c) HEK293 cells transfected with Myc-PEX5-WT and HA-Ub for 24 h following a prior siRNA knockdown of ATM for 48 h, were treated with H₂O₂ for 6 h and immunoprecipitated with anti-HA and immunoblotted with anti-Myc antibodies. Input lysates were immunoblotted using the indicated antibodies. (d) HEK 293 cells were transfected with siRNA for PEX2, PEX10 and PEX12, and treated with H₂O₂ for 6 h. Immunoprecipitation was performed with an anti-PEX5 antibody followed by immunoblotting for endogenous ubiquitin. RT-PCR was performed to confirm siRNA knockdown of PEX5, PEX10 and PEX12. (e) HEK293 cells transfected with Myc-PEX5-WT/Myc-PEX5-K209R and HA-Ub constructs for 24 h following a prior siRNA knockdown of PEX2/10/12 for 48 h were immunoprecipitated with anti-HA and immunoblotted with anti-Myc antibodies. Input lysates were immunoblotted using the indicated antibodies. Uncropped images of western blots are shown in Supplementary Fig. S9.
Supplementary Figure S6  K209 is responsible for PEX5 interaction with p62 in response to ROS. (a) Representative image of HepG2 cells treated with or without H$_2$O$_2$ for 3 h and immunostained for FIP200 or ATG16L (green) and peroxisomal marker PMP70 (red). Scale bar, 10 μm. High-magnification images of boxed areas showed co-localization between FIP200 or ATG16L and PMP70 (Scale bar, 3 μm). (b) HEK293 cells were transfected with Myc-PEX5-WT and HA-p62, and treated with 0.4 mM of H$_2$O$_2$ for indicated times. The complex of HA-p62 and Myc-PEX5 was immunoprecipitated with anti-HA and immunoblotted with anti-Myc. Inputs were immunoblotted using the indicated antibodies. (c) HEK293 cells were transfected with HA-p62 and Myc-PEX5-WT or Myc-PEX5-K209R. The complex of HA-p62 and Myc-PEX5 was immunoprecipitated with anti-HA and immunoblotted with anti-Myc antibodies. Input lysates were immunoblotted using the indicated antibodies. Uncropped images of western blots are shown in Supplementary Fig. S9.
Supplementary Figure S7 Regulation of pexophagy by ROS is ATM-dependent. (a) Frame-capture from time course imaging of pexophagy in live RPE cells stably expressing GFP-LC3 and DsRed-SKL following H2O2 (0.4 mM) treatment. Scale bar, 10 μm. (b) Western analysis of HepG2 cells pre-incubated in the presence or absence of Bafilomycin A1 (Baf A1, 200 nM) for 1 h before treatment with 0.4 mM H2O2 for 3 h using anti-PEX1, anti-PEX14, anti-p62 and LC3-II antibodies. (c) Quantification of PEX1 and PEX14 intensity normalized to GAPDH from Fig. S7b (mean ± s.d., n = 3 independent experiments, Student’s t test). * P < 0.05, ** P < 0.01, *** P < 0.001, NS = not significant. (d) Western analysis of whole cell extracts (WCE) and peroxisome fractions (P) of HepG2 cells treated with Baf A1 (200 nM) for 4 h immunoblotted using anti-ATM, anti-pPEX5(S141) and PEX5 antibodies. (e) Western analysis of HepG2 cells transfected with or without siP62 for 72 h and treated with 0.4 mM H2O2 for 3 h using anti-PEX1, anti-PEX14, anti-pATM, anti-ATM, anti-p62 and LC3-II antibodies. (f) Quantification of PEX1 and PEX14 intensity normalized to GAPDH from Fig. S7e (mean ± s.d., n = 3 independent experiments, Student’s t test). ** P < 0.01, *** P < 0.001, NS = not significant. (g) Representative images of wild type (WT, GM08399) and ataxia telangiectasia (AT, GM05849) patient fibroblasts treated with H2O2 for 6 h, and immunostained with PEX14 antibody. Scale bar, 15 μm. (h) Quantification of PEX14 puncta per cell was performed from Fig. S7g from n = 4 independent experiments, 100 cells were analyzed in each experiment. All error bars represent s.d., (Student’s t test) *** P < 0.001, NS = not significant. (i) Western analysis of AT (GM05849) fibroblasts transfected with Flag-ATM WT or Flag-ATM KD (kinase dead) mutant and treated with 0.4 mM H2O2 for 6 h using anti-PEX1, anti-PEX14, anti-pATM, anti-Flag and GAPDH antibodies. (j) Western analysis of HEK293 cells transfected with Pex2/10/12 siRNA for 72 h and treated with H2O2 (0.4 mM) for 6 h using anti-PEX1, anti-PEX14, anti-pATM, ATM and GAPDH antibodies. Uncropped images of western blots are shown in Supplementary Fig. S9. Statistic source data for Supplementary Fig. S7 c, f, h can be found in Supplementary Table 1.
Supplementary Figure S8   Pexophagy in HepG2 cells with H$_2$O$_2$. Representative electron microscopy images of HepG2 cells treated with vehicle or H$_2$O$_2$. (a) Vehicle 6 h, (b, c and d) H$_2$O$_2$ 6 h (0.4 mM). Scale bar, 0.5 μm. Autophagosomes containing peroxisome are represented by yellow arrows, and shown enlarged in the boxed area (Scale bar, 0.25 μm).
Supplementary Figure S9  Uncropped images of all the western blots.
Supplementary Figure S9 continued
Supplementary Figure S9 continued
Supplementary Figure S9 continued
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Supplementary Figure S9 continued
Table 1 Source data table. Details of all the quantitative raw data values, sample size and the statistical test applied.
Supplementary video legends

Supplementary Video 1  Formation of autophagosomes over peroxisome. RPE cells stably expressing GFP-LC3 and DsRed-SKL and treated with H₂O₂ (0.4 mM, from 30min to 3hr) imaged by time-lapse microscopy.

Supplementary Video 2: Induction of pexophagic flux with H₂O₂. MCF7 cells transfected with mRFP-EGFP-SKL and treated with H₂O₂ (0.4 mM, from 30min to 3hr) imaged by time-lapse microscopy.