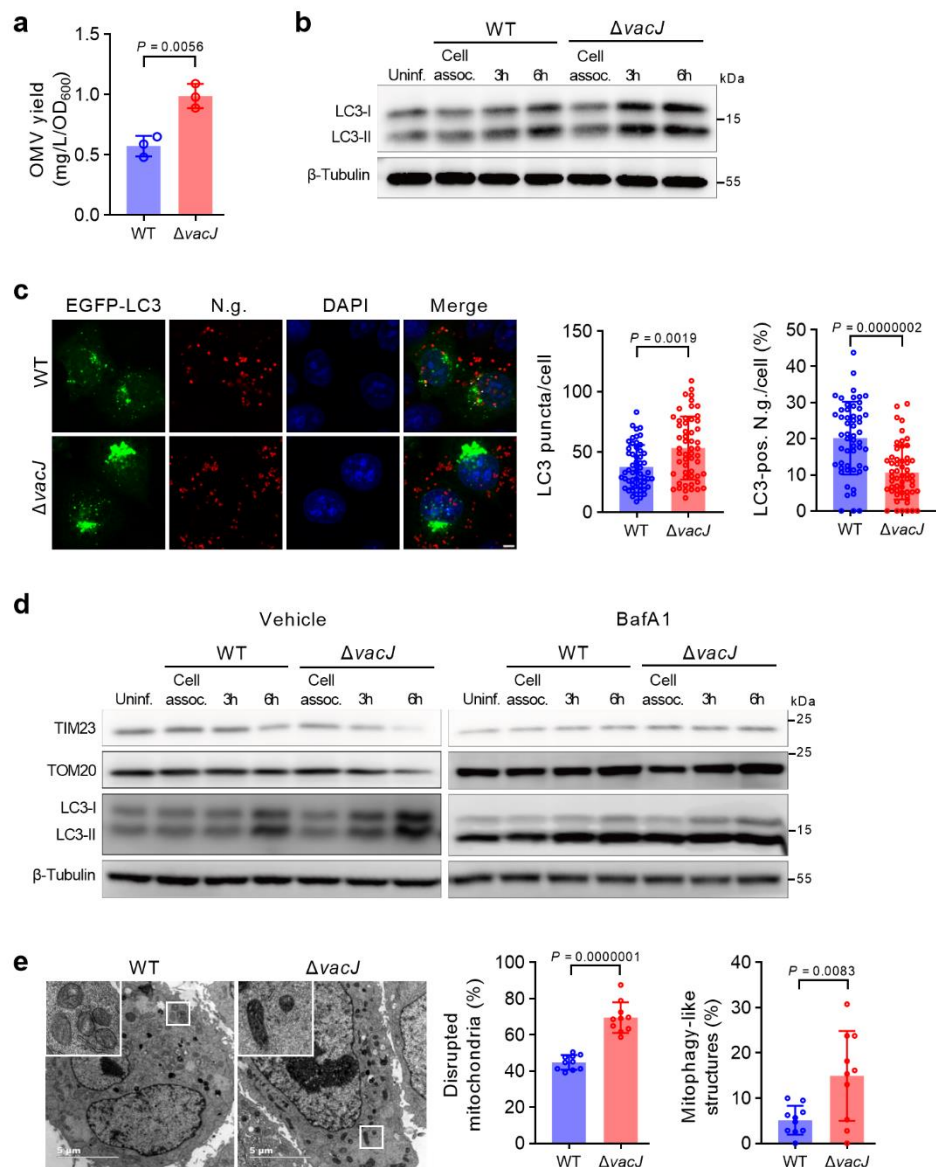


Gonococcal OMV-delivered PorB induces epithelial cell mitophagy

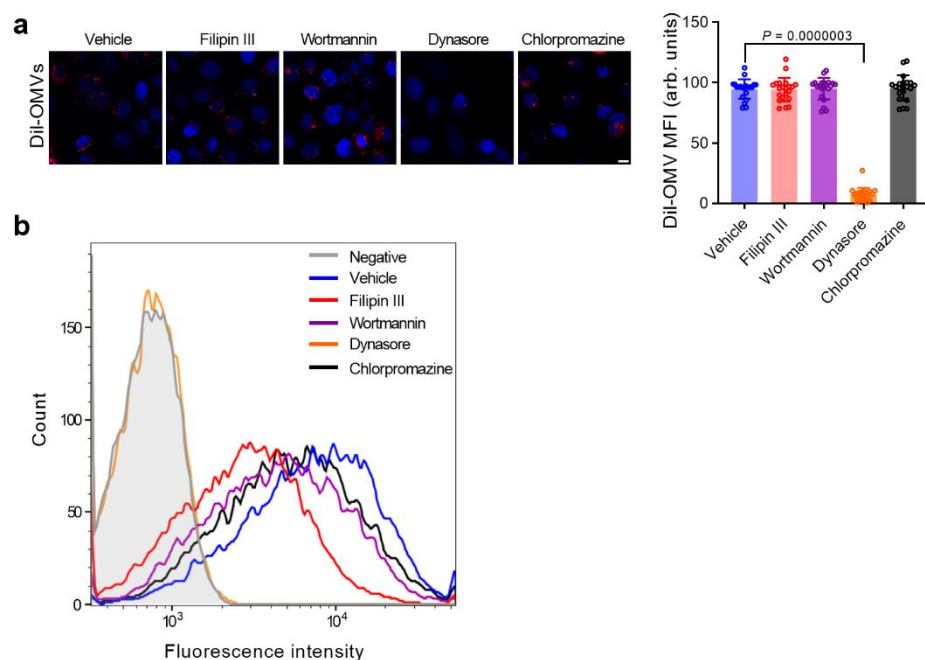
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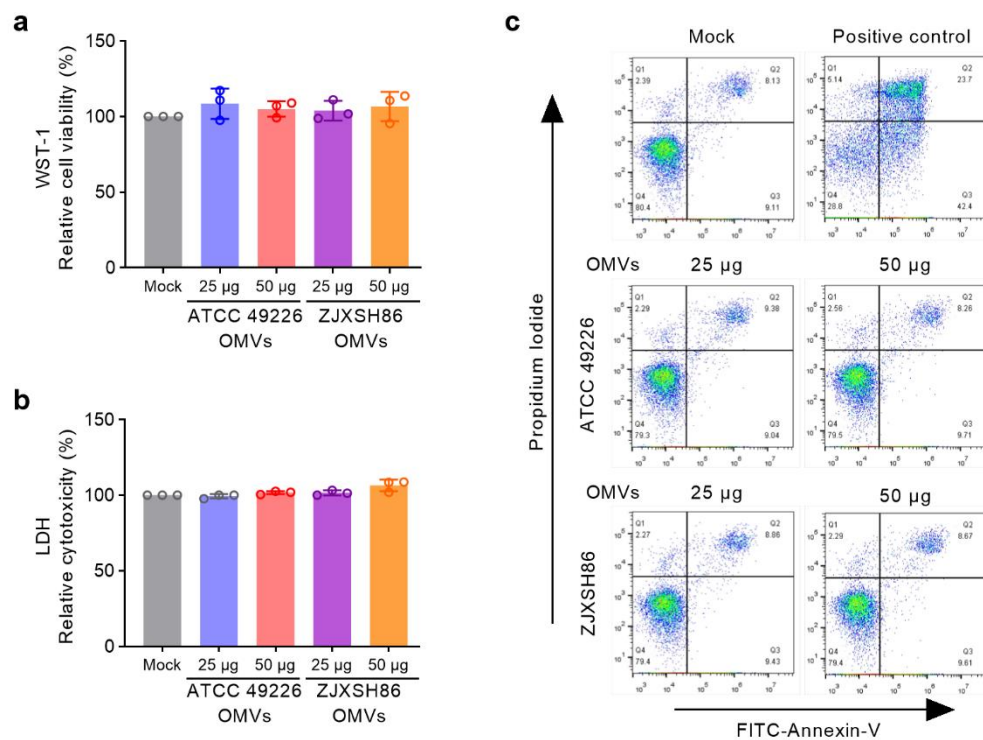


Supplementary Fig. 1 | The gonococcal $\Delta vacJ$ mutant shows enhanced OMV secretion and increased induction of mitophagy. **a** Quantification of OMV yields based on total proteins after OMV purification shows enhanced OMV yields obtained for the *Neisseria gonorrhoeae* $\Delta vacJ$ mutant. Data are mean $\pm$ s.d.; $n = 3$ independent experiments, unpaired two-tailed t -test. **b** LC3 Western blots after gentamicin protection assays show enhanced LC3 accumulation for the $\Delta vacJ$ mutant. **c** Microscopy images showing increased accumulation of total LC3 puncta for the $\Delta vacJ$ mutant after a one-hour challenge of HeLa cells, but reduced colocalization between the $\Delta vacJ$ mutant and LC3 puncta. Scale bar, 5 μ m. Data are mean $\pm$ s.d.; $n = 54$ cells from 3 independent experiments, two-tailed Mann-Whitney test for quantification of LC3 puncta, unpaired two-tailed t -test for quantification of colocalized LC3 puncta. **d** Western blots of LC3 and mitochondrial marker proteins TOM20 and TIM23 after gentamicin protection assays with vehicle control- or BafA1-pretreated HeLa cells show enhanced mitochondrial degradation and autophagy flux for the $\Delta vacJ$ mutant. **e**

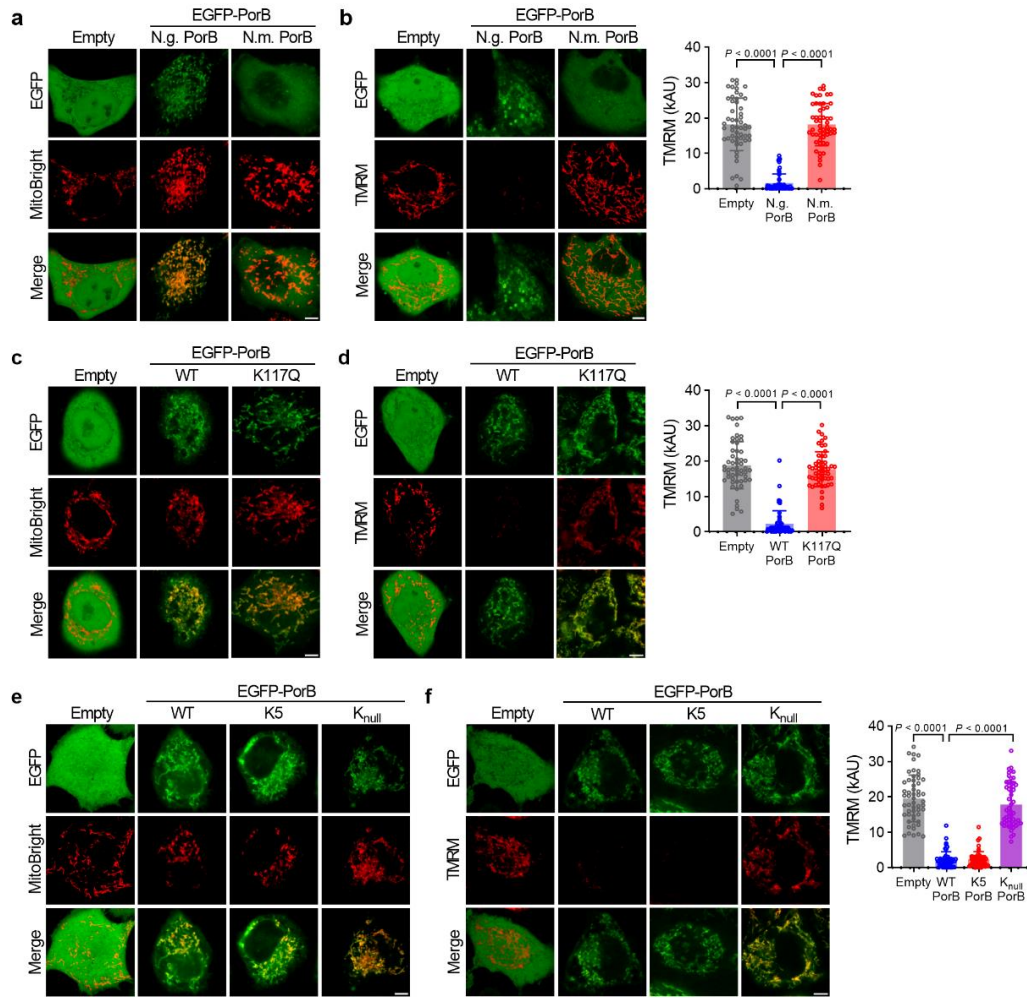
TEM images showing that a challenge of HeLa cells with *N. gonorrhoeae* results in increased mitochondrial disruption and mitochondrial capture in mitophagy-like structures for the $\Delta vacJ$ mutant (one hour after addition of gentamicin). Data are mean $\pm$ s.d.; $n = 10$ cells from 2 independent experiments, unpaired two-tailed *t*-test. Western blots in **b**, **d** are representative of 3 independent experiments. Source data are provided as a Source Data file.



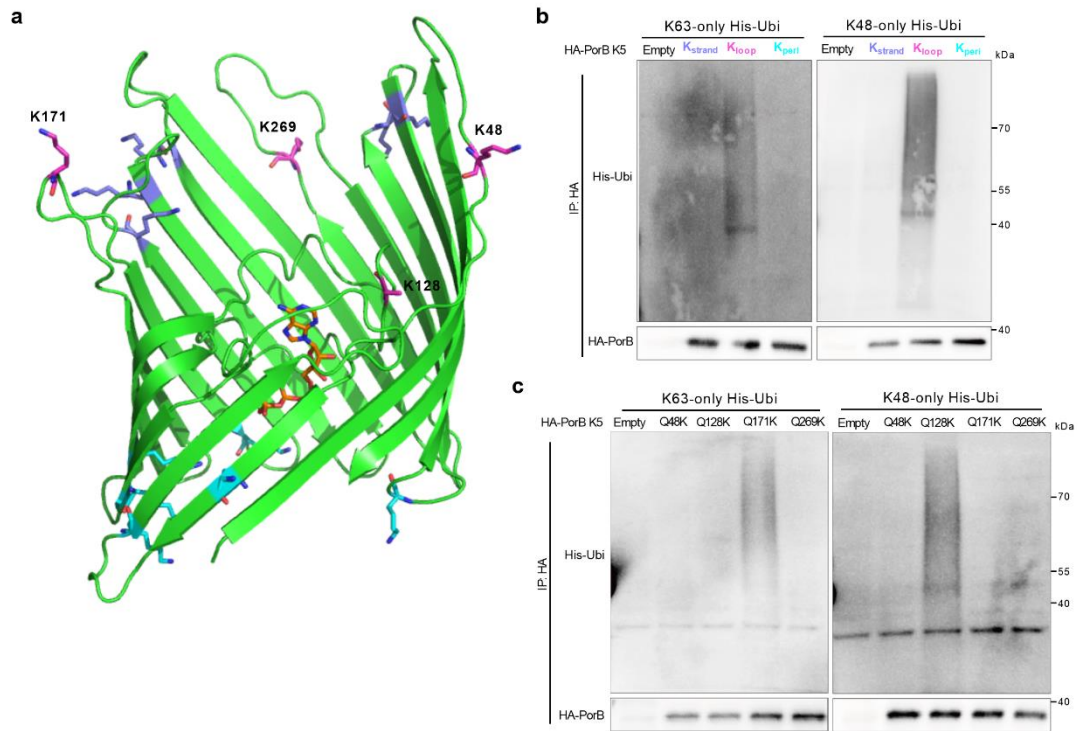
Supplementary Fig. 2 | Gonococcal OMV endocytosis in epithelial cells is dynamin dependent. **a** Microscopy images showing OMV endocytosis of HeLa cells is inhibited by the dynamin inhibitor Dynasore, and not by inhibitors of caveolin/lipid rafts (filipin III), macropinocytosis (wortmannin) or clathrin (chlorpromazine). Scale bar, 5 μ m. Data are mean $\pm$ s.d.; $n = 21$ cells from 3 independent experiments, Kruskal-Wallis with posthoc Dunn test. **b** Flow cytometry analysis of Dil-OMV fluorescence intensity after endocytosis of HeLa cells showing endocytosis is dynamin-dependent. Source data are provided as a Source Data file.



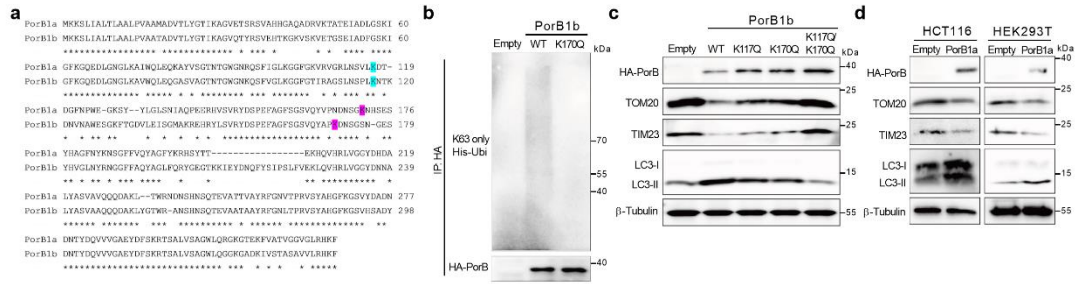
Supplementary Fig. 3 | Gonococcal OMVs do not affect cellular viability or induce cellular apoptosis. **a** Determination of HeLa cell viability with the WST-1 assay shows that OMV endocytosis does not affect cellular viability. Data are mean $\pm$ s.d.; $n = 3$ independent experiments. **b** Determination of OMV cytotoxicity with the LDH assay shows that OMVs do not display cytotoxicity after endocytosis of HeLa cells. Data are mean $\pm$ s.d.; $n = 3$ independent experiments. **c** Flow cytometry analysis of HeLa cells to detect for apoptosis using FITC-Annexin V/propidium iodide shows that OMV endocytosis does not induce apoptosis. Source data are provided as a Source Data file.



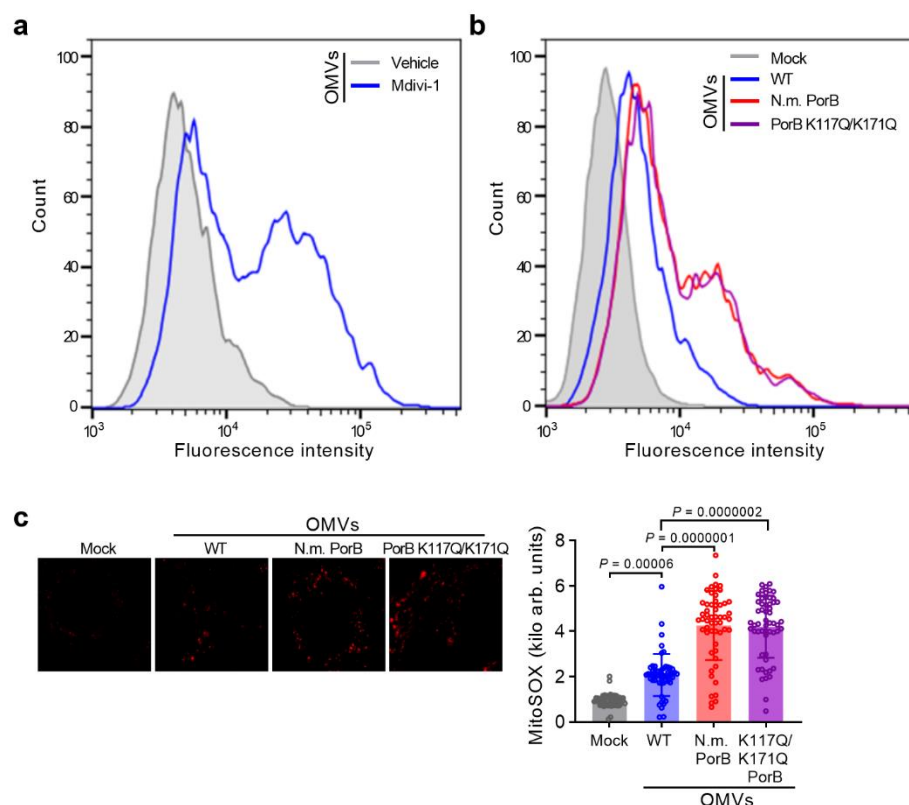
Supplementary Fig. 4 | Gonococcal PorB targets mitochondria and dissipates the mitochondrial membrane potential. **a** Representative microscopy images showing gonococcal PorB, but not PorB from *Neisseria mucosa*, localizes at HeLa cell mitochondria. Scale bar, 5 μ m. **b** Representative microscopy images showing gonococcal PorB, but not PorB from *N. mucosa*, dissipates the mitochondrial membrane potential (MMP). Scale bar, 5 μ m. Data are mean $\pm$ s.d.; $n = 54$ cells, Kruskal-Wallis with posthoc Dunn test. **c** Representative microscopy images showing that the gonococcal PorB K117Q mutant localizes at HeLa cell mitochondria. Scale bar, 5 μ m. **d** Representative microscopy images showing that the gonococcal PorB K117Q mutant is unable to dissipate MMP. Scale bar, 5 μ m. Data are mean $\pm$ s.d.; $n = 54$ cells, Kruskal-Wallis with posthoc Dunn test. **e** Representative microscopy images showing that the gonococcal PorB K5 (only retaining the 5 lysines associated with ATP binding in the PorB channel) and K_{null} (all lysines replaced by glutamines) mutants localize at HeLa cell mitochondria. Scale bar, 5 μ m. **f** Representative microscopy images showing that gonococcal PorB K5 dissipates MMP, while PorB K_{null} lost this ability. Scale bar, 5 μ m. Data are mean $\pm$ s.d.; $n = 54$ cells, Kruskal-Wallis with posthoc Dunn test. Images in **a**, **c**, **e** are representative of 3 independent experiments. Cells in **b**, **d**, **f** are from 3 independent experiments. $P < 10^{-15}$ for all reported values. Source data are provided as a Source Data file.



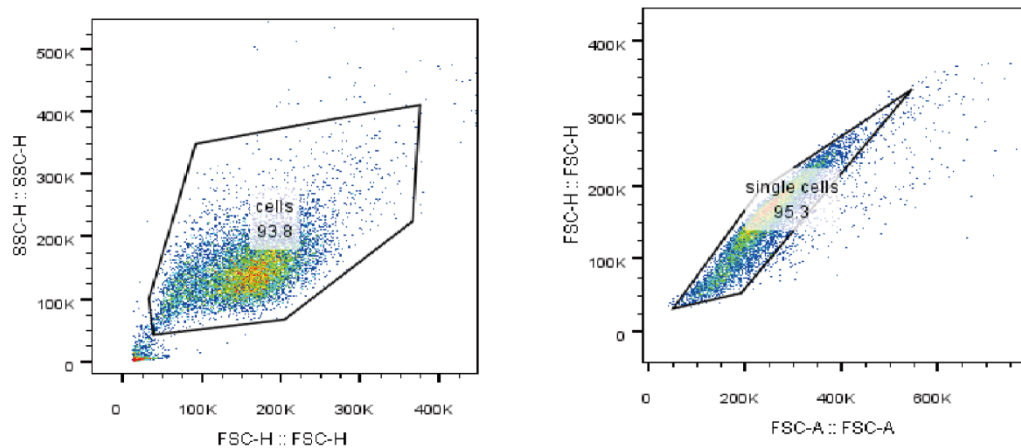
Supplementary Fig. 5 | Gonococcal PorB lysine 171 is decorated with K63-linked polyubiquitin. **a** Side-view of the gonococcal PorB structure (pdb entry 4AUI) with lysine residues retained by the PorB K_{strand} mutant displayed in purple, lysine residues retained by the PorB K_{loop} mutant displayed in magenta, and lysine residues retained by the PorB K_{peri} mutant displayed in blue. **b** Western blots after immunoprecipitation of HA-PorB K5 (only retaining the five lysine residues associated with ATP binding in the PorB channel)-derivatives K_{strand} , K_{loop} , and K_{peri} from HeLa cells show co-immunoprecipitation of both ubiquitin that can only form K63-linked or only K48-linked polyubiquitin for the PorB K_{loop} mutant. **c** Western blots after immunoprecipitation of HA-PorB K5-derivatives Q48K, Q128K, Q171K, and Q269K show co-immunoprecipitation of K63-linked polyubiquitin for the PorB K5-Q171K mutant and co-immunoprecipitation of K48-linked polyubiquitin for the PorB K5-Q128K mutant. Western blots in **b**, **c** are representative of 3 independent experiments.



Supplementary Fig. 6 | Gonococcal PorB-induced mitophagy is not restricted to specific PorB type or epithelial cell types. **a** Amino acid sequence alignment of PorB1a from *Neisseria gonorrhoeae* strain ATCC 49226 and PorB1b from strain ZJXSH86, which share 70% sequence identity. PorB lysine residue 117 is highlighted in blue, and PorB1a lysine 171 and PorB1b lysine 170 are highlighted in magenta. **b** Western blots after immunoprecipitation of HA-PorB1b WT and the K170Q mutant from HeLa cells show that co-immunoprecipitation of ubiquitin that can only form K63-linked polyubiquitin is dependent on PorB1b lysine residue 170. **c** Western blots showing PorB1b-induced accumulation of LC3 and degradation of TOM20 and TIM23 in HeLa cells is dependent on PorB1b lysines 117 and 170, with degradation fully inhibited for a PorB1b K117Q/K170Q double mutant. **d** Western blots showing PorB1a-induced accumulation of LC3 and degradation of TOM20 and TIM23 in HEK293T and HCT116 cells. Western blots in **b**, **c**, **d** are representative of 3 independent experiments.



Supplementary Fig. 7 | Gonococcal OMVs induce mitophagy to reduce generation of mitochondrial ROS. **a** Flow cytometry analysis of fluorescence intensity of the ROS-sensitive MitoSOX probe after HeLa cell endocytosis of gonococcal OMVs shows that pretreatment with mitophagy inhibitor Mdivi-1 prevents OMV-induced reduction of mitochondrial ROS levels. **b** Flow cytometry analysis of fluorescence intensity of MitoSOX shows that OMVs expressing PorB K117Q/K171Q or PorB from *Neisseria mucosa*, which are unable to induce mitophagy, are not able to reduce mitochondrial ROS levels after HeLa cell endocytosis. **c** Live-cell microscopy images of ROS levels in OMV-stimulated HeLa cells show that OMVs expressing gonococcal PorB K117Q/K171Q or PorB from *N. mucosa* are unable to reduce mitochondrial ROS levels after HeLa cell endocytosis. Scale bar, 5 μ m. Data are mean $\pm$ s.d.; $n = 55$ cells from 3 independent experiments, Kruskal-Wallis with posthoc Dunn test. Source data are provided as a Source Data file.



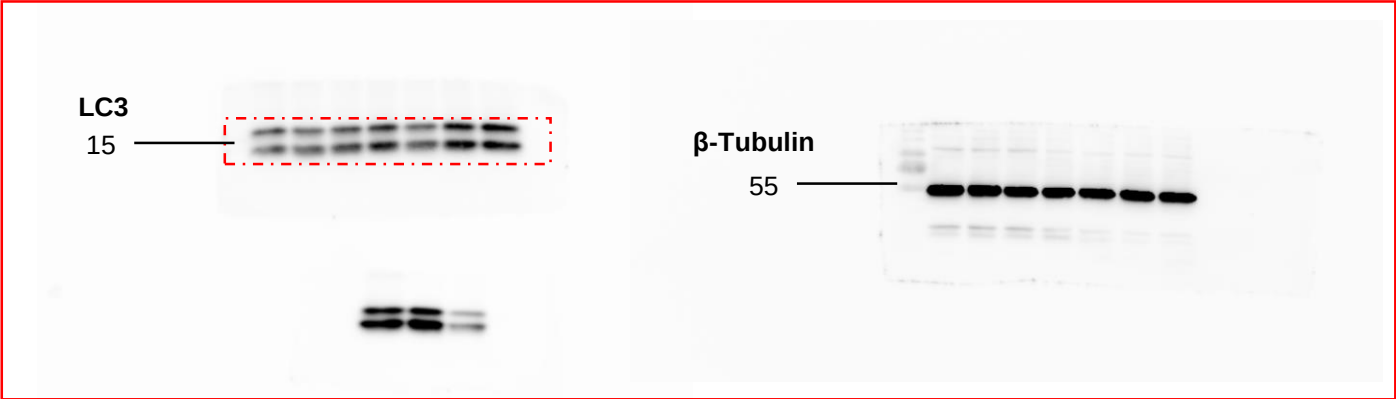
Supplementary Fig. 8 | Gating strategy. Representative plots showing selection of cells based on forward scatter height (FSC-H) and side scatter height (SSC-H), and single cells were selected based on FSC-H and forward scatter area (FSC-A) to exclude doublets.

Supplementary Table 1 | Primers used in this study.

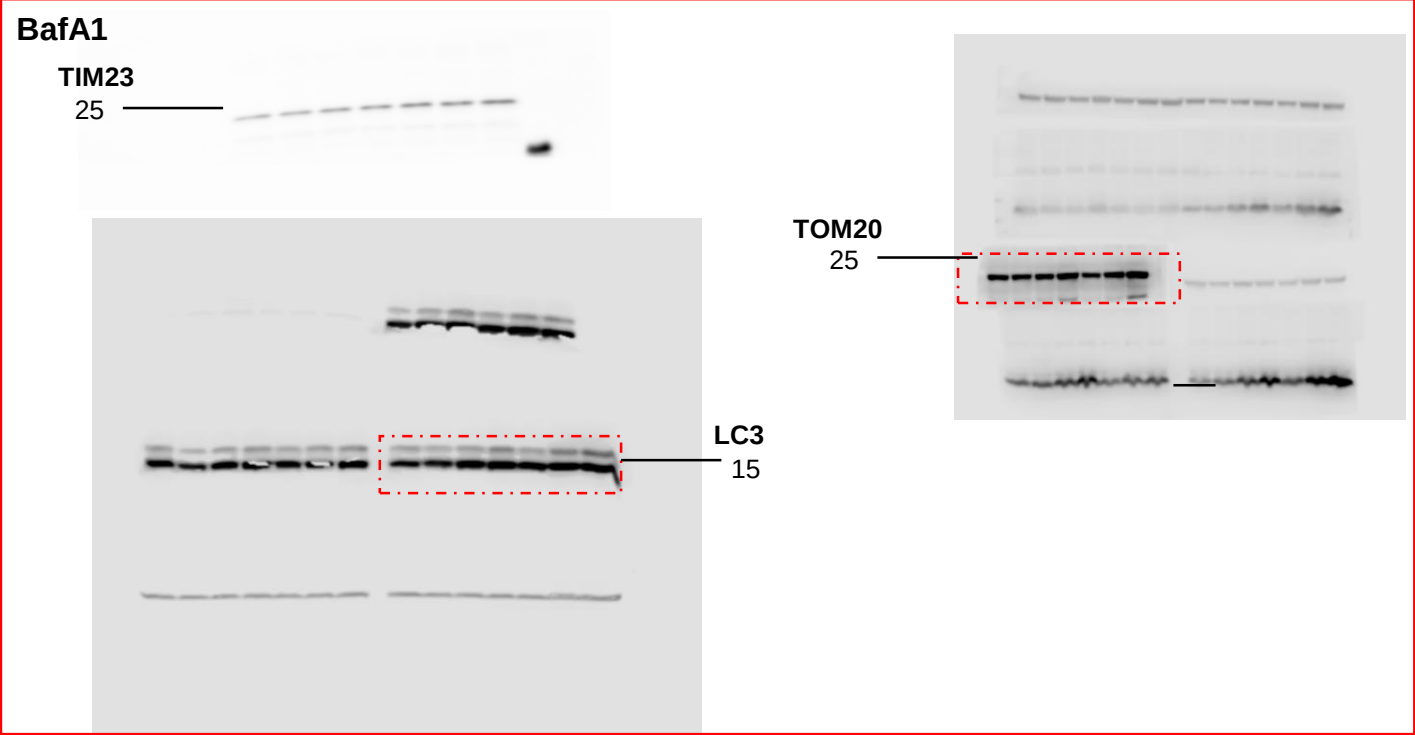
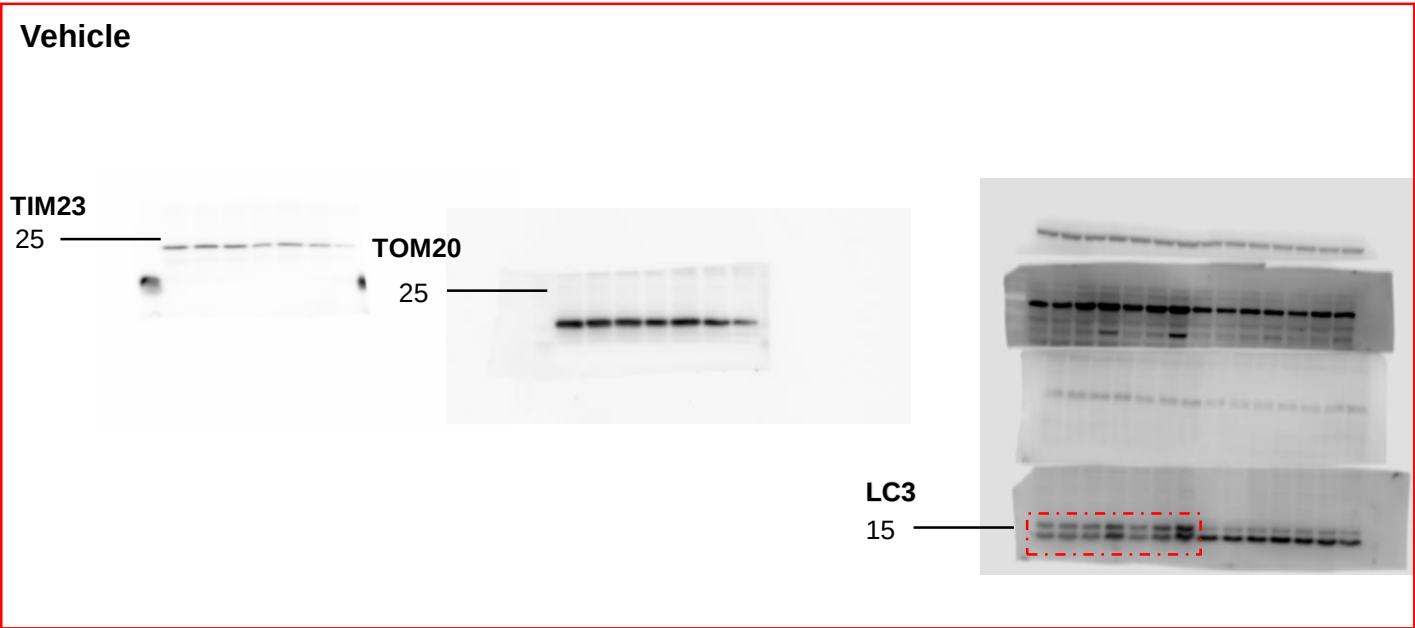
Primer name	Primer sequence 5'-3'
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vacJ-upR	GCGAATTCGATTTTCAGCCAGTCTTTGA
vacJ-downF-1	GCGAATTCAGCCATGCCGTCTGAAAAT
vacJ-downR-1	GCTCTAGAGTAAACGGCAGTCCGTAGT
vacJ-downF-2	GCAGATCTAGCCATGCCGTCTGAAAAT
vacJ-downR-2	GCGTCGACGTAAACGGCAGTCCGTAGT
ldcA-UF	GCGAGCTCCAGACGGCATTGTTCTCCC
ldcA-UR	GCTCTAGAAATTTCCCTTTCATACGGT
ldcA-DF	GCGGATCCACCCGCAAACGGACAAAATG
ldcA-DR	GCCTGCAGCTGCTGGCAATCTTTGTCTG
coxII-q-F	GCGCAAGTAGGTCTACAAG
coxII-q-R	GGCAGGATAGTTCAGACGG
human RPL13A-F	CGCCCTACGACAAGAAAAAG
humanRPL13A-R	CCGTAGCCTCATGAGCTGTT
porB-F	GCGAATTCATGAAAAAATCCCTGATTGCCC
porB-R	GCGGATCCTTAGAATTTGTGGCGCAGAC
porB(2)-F	GCGAATTCATGAAAAAATCCCTGATTGCCC
porB(2)-R	GCGGATCCTTAGAATTTGTGGCGCAGAA
porB-K117Q-F	TTGAACAGCGTCTCTGCAAGACACCGACGGCTTCAAT
porB-K117Q-R	GAAGCCGTCGGTGTCTTGCAGGACGCTGTTCAAACG
PorB-Q48K-F	GCGGATCGCGTTAAAACCGCTACCGAAATCGCT
PorB-Q48K-R	TTCGGTAGCGGTTTTAACGCGATCCGCCTGAGC
PorB-Q128K-F:	CCTTGGGAGGGTAAAAGCTACTATTTGGGTTTA
PorB-Q128K-R	CAAATAGTAGCTTTTACCCTCCCAAGGATTGAA
PorB-Q171K-F	GACAATTCGGGCAAAAATCACAGCGAATCTTAC
PorB-Q171K-R	TTCGCTGTGATTTTTGCCCCGAATTGTCGTTAGG
PorB-Q269K-F:	GCCCACGGCTTCAAAGGTTTCGGTTTATGATGCA
PorB-Q269K-R	ATAAACCGAACCTTTGAAGCCGTGGGCGTAAGA
PorB-K128Q-F	CCTTGGGAGGGTCAAAGCTACTATTTGGGTTTA
PorB-K128Q-R	CAAATAGTAGCTTTGACCCTCCCAAGGATTGAA
PorB-K171Q-F	GACAATTCGGGCCAAAATCACAGCGAATCTTAC
PorB-K171Q-R	TTCGCTGTGATTTTTGCCCCGAATTGTCGTTAGG
PorB(2)-K117Q-F	CTGAACAGCCCCCTGCAAAACACC
PorB(2)-K117Q-R	GTTGTCCTTGGTGTTCCTGCAGGGG
PorB(2)-K170Q-F	GTACAATACGCACCTCAAGATAAT
PorB(2)-K170Q-R	TGAGCCTGAATTATCTTGAGGTGC
dus-mucosa-F	ATGCCGTCTGAAATGCCGTCTGAAGGTTTGCAA
dus-mucosa-R	TTCAGACGGCATTTCAGACGGCATTGACGGAAG
dus-porB-F	ATGCCGTCTGAAGAATTCCCAATTTCGTACGCT
dus-porB-R	TTCAGACGGCATAAGCTTTGACGGAAGGCAAC

Supplementary Figures: Uncropped Western blots

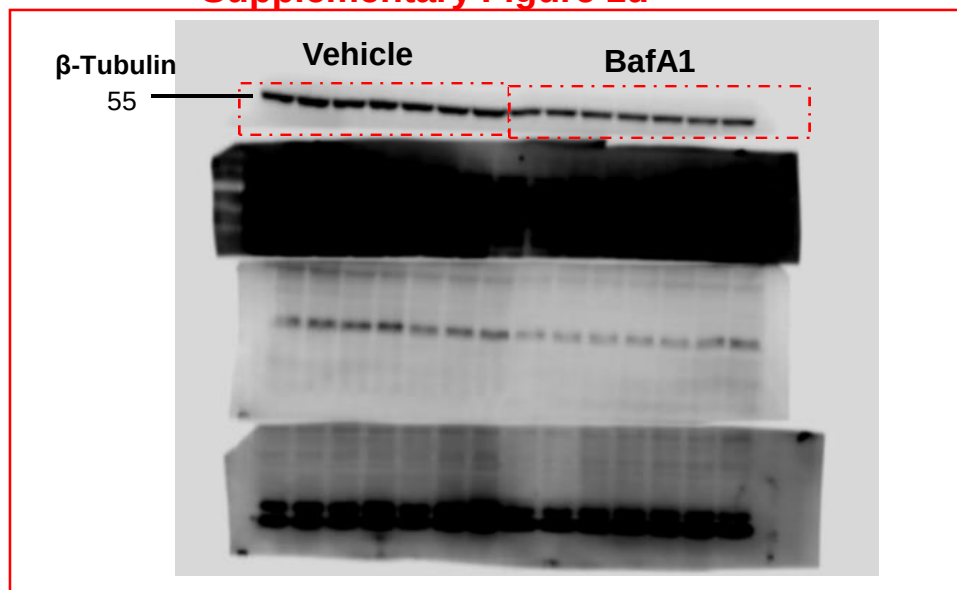
Supplementary Figure 1b



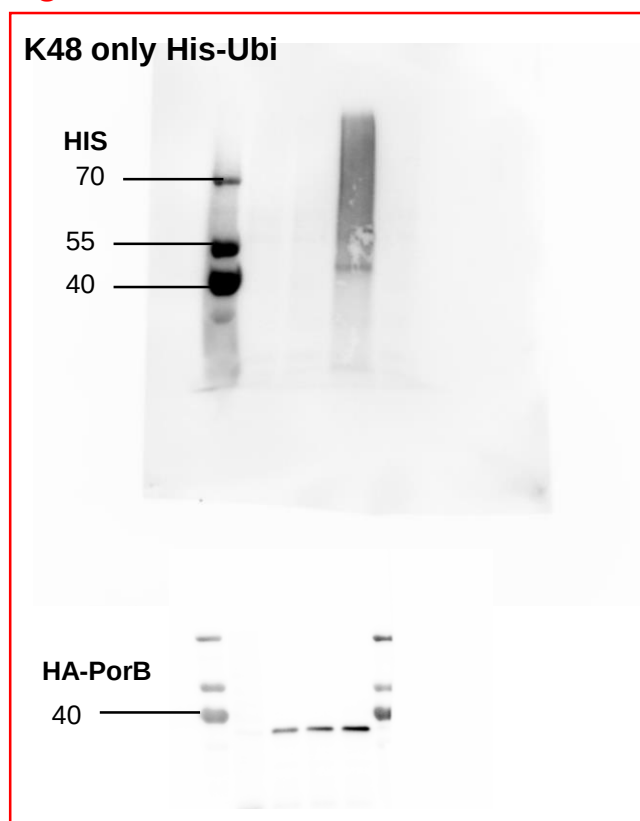
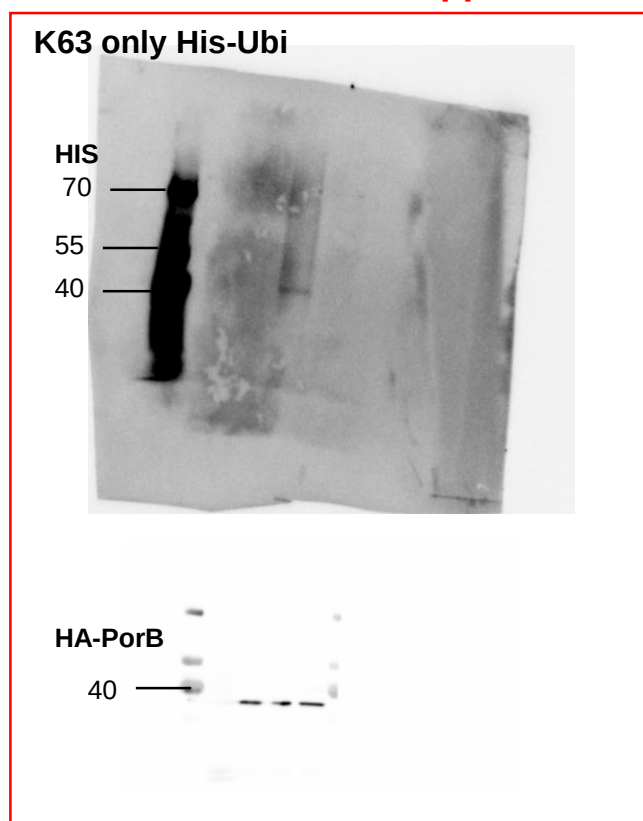
Supplementary Figure 1d



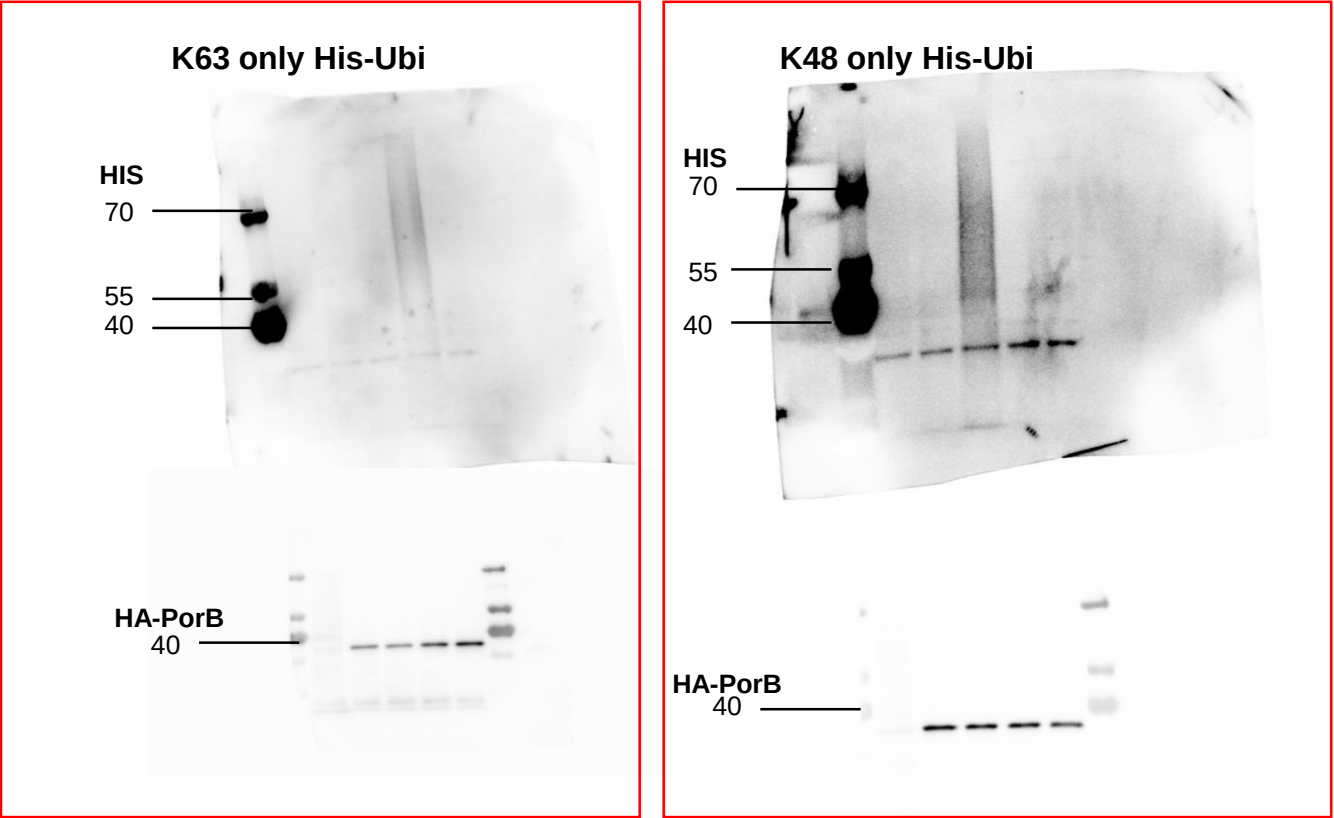
Supplementary Figure 1d



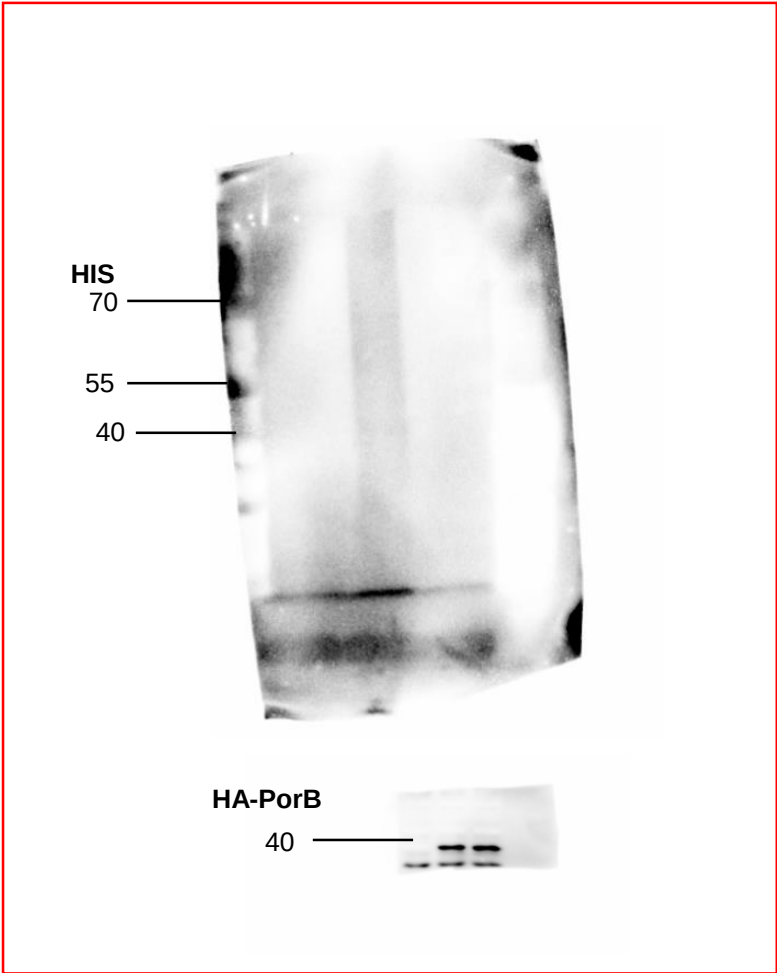
Supplementary Figure 5b



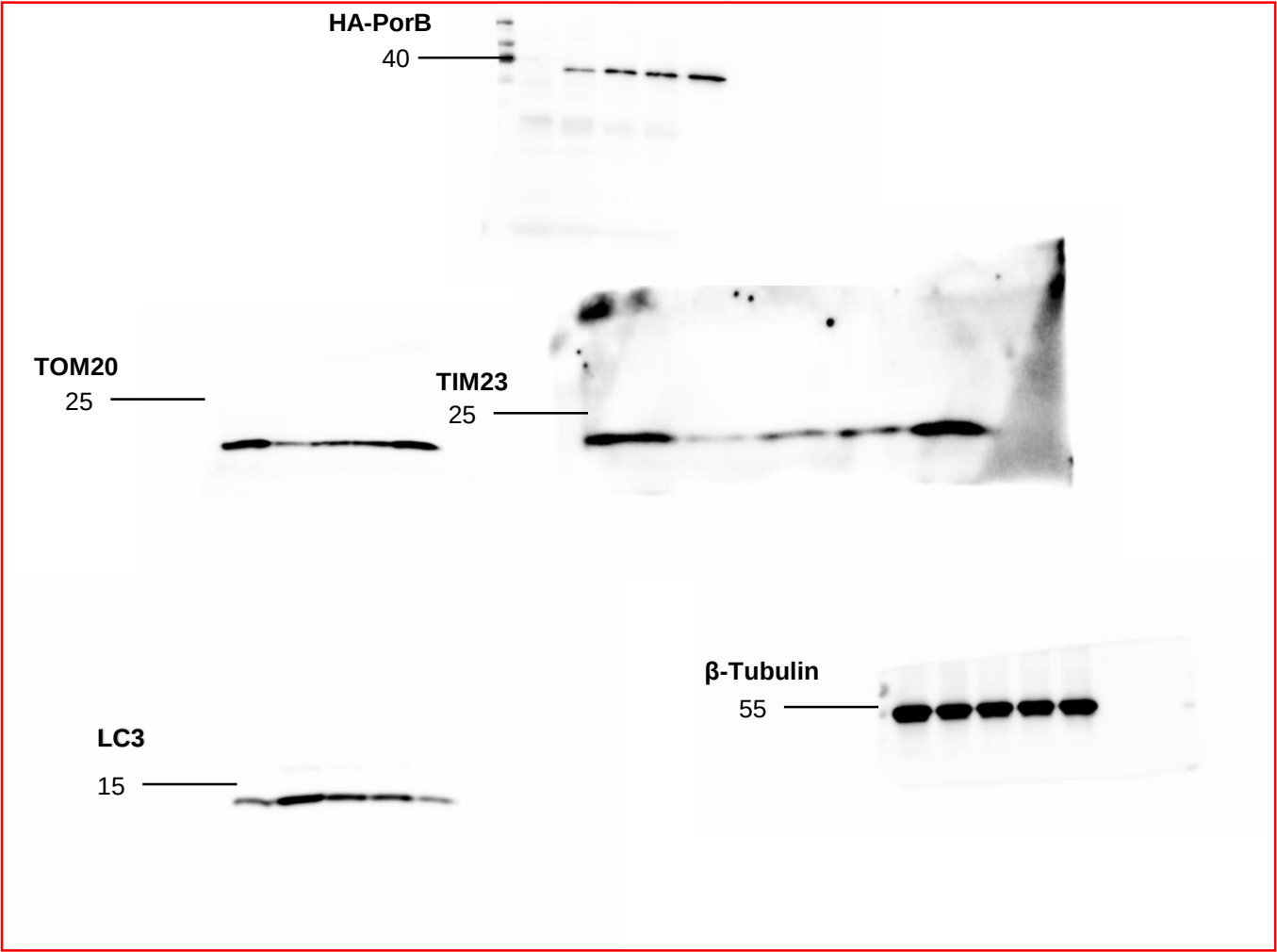
Supplementary Figure 5c



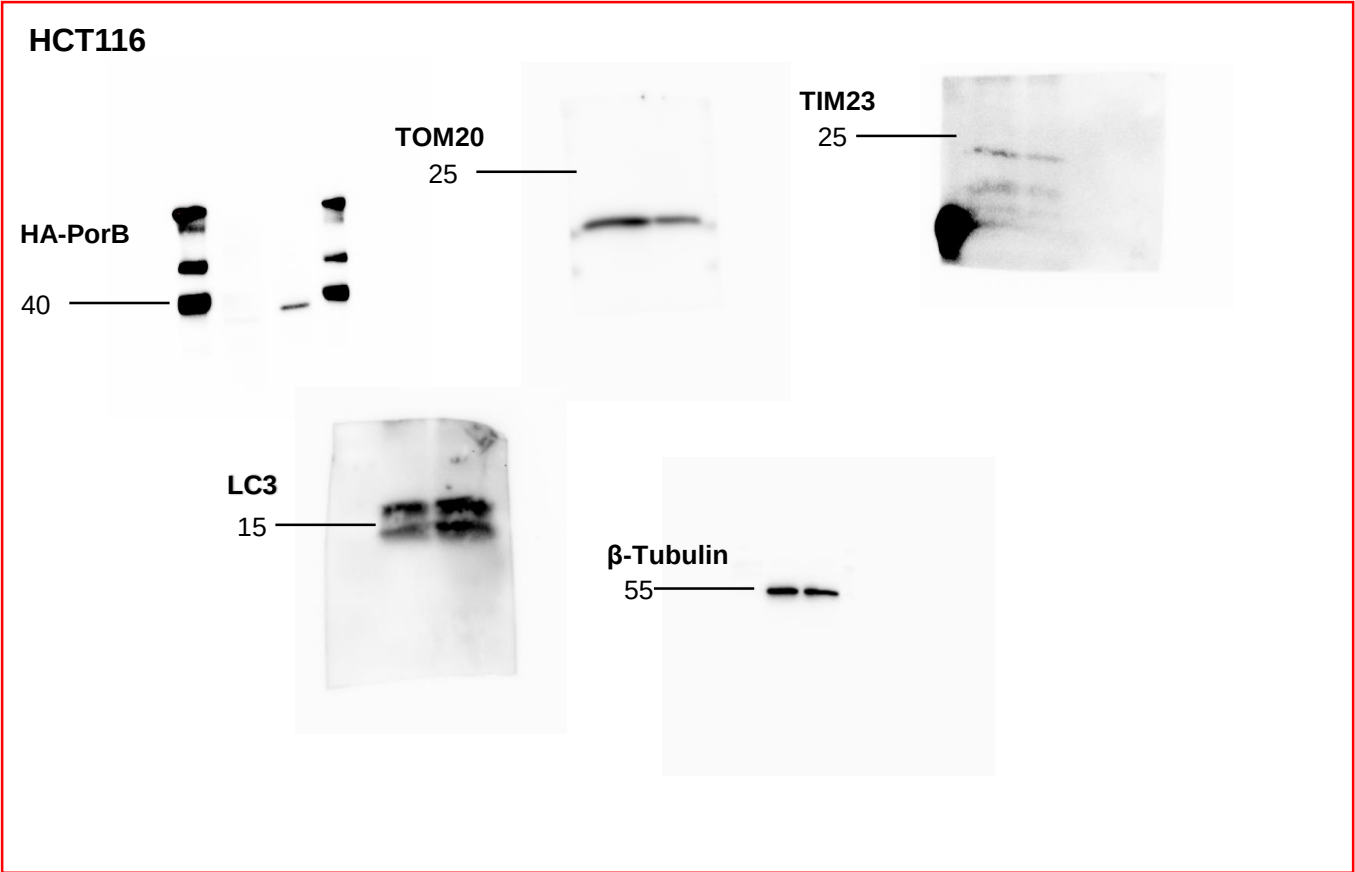
Supplementary Figure 6b



Supplementary Figure 6c



Supplementary Figure 6d



Supplementary Figure 6d

