Martinez et al. “Molecular characterization of LC3-associated phagocytosis (LAP) reveals distinct roles for Rubicon, NOX2, and autophagy proteins.”

Materials and Methods

Generation of Rubicon-deficient mice using CRISPR/Cas9 system

Rubicon-deficient mice were generated by using CRISPR/Cas9 technology as described previously[55], with minor modifications[56]. A premature STOP codon replacing Asp178 was introduced by homology directed repair (HDR) following injection of pronuclear stage zygotes with capped Cas9 mRNA transcripts, an sgRNA targeting the end of exon 5 and a 183 nucleotide-long single stranded DNA oligonucleotide that served as template for HDR (Rubicon-HDR, IDT, Ultramer Technology). To facilitate the identification of founder mice and genotyping, additional substitutions generating an EcoRI restriction site were introduced downstream of the stop codon. The sequence of Rubicon-HDR is:

5’-

GCCTTCCTGCTGAGTGACGCCCACGTCACAGCCATGCTCCAGTGCCTGGAAGCAG

TGGAACAGAACAACCCCCGTCTTCTGGCTCAGATCtAaGaATtCA**TGG**TAAAAGGGG

CGAAGGATTGCCCGTGTCTGTCCTGGCCTCTCCACTTCACCGTGATGTGCATGCCA

CGGGAGCCCATGACT-3’. Mutated bases are shown in lower-case letters. The PAM sequence is shown in bold. The sgRNA target sequence is underlined and the premature STOP codon is boxed. In accordance with the guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/), the nomenclature for the mutation is: NM_001200038.1:c.562_569delinsTAAGAATT.

Capped Cas9 mRNA transcripts were produced from PmeI-lineralized and column-purified pcDNA3.3topo-T7-hCas9[56] using mMESSAGE mMACHINE T7 ULTRA Kit (Life

Technologies). The pcDNA3.3topo-T7-hCas9 plasmid was created by adding a T7 promoter in the XbaI site of a plasmid, hCas9 (Addgene 41815), encoding a human codon optimized Cas9 nuclease.

For the guide RNA, a target site (Rubicon-E5F2) toward the ends of exon 5 was selected (Rubicon-E5F2, GGCTCAGATCGATGCATCCA; position, chr16:32,847,305-32,847,324 in NCBI37/mm9 mouse assembly). The specificity of this sequence and the absence of off-target activity were confirmed (see Supplementary Table 2). The Rubicon-E5F2-F and Rubicon-E5F2-R primers (see Supplementary Table 3) were annealed and cloned in the gRNA_Cloning Vector (Addgene: 41824) to generate the pCR-Blunt-II-U6-gRNA-Rubicon-E5F2 plasmid. This plasmid was used as template to add a T7 promoter to the gRNA sequence by PCR amplification with the T7-Rubicon-E5F2 and gRNA-R (see Supplementary Table 3). The PCR product was column-purified by QIAquick PCR Purification (QIAGEN) and used as the template to produce the sgRNA transcript using MEGAshortscript T7 Kit (Life Technologies).

The Cas9 mRNA and the sgRNA were purified using MEGAclear kit (Life Technologies, Carlsbad, CA) and eluted in nuclease-free water. The integrity of the Cas9 mRNA and the gRNA was confirmed by analysis on a 2100 Bioanalyzer (Agilent Technologies).

Zygotic injection of CRISPR/Cas9 and Rubicon HDR was performed by The Transgenic Gene Knockout Shared Resource (St. Jude Children's Research Hospital). Zygotes were obtained from super-ovulated C57BL/6N female mated to C57BL/6J stud males. A mixture of Cas9 mRNA (100 ng/μl), sgRNA (50 ng/μl) and oligonucleotides (2 pmol/μl) was injected into the cytoplasm of zygotes at the pronuclei stage. Approximately 25 injected zygotes were the transferred to the oviduct of pseudopregnant CD1 females.

The genomes of mice obtained from zygotic injections were analyzed by i) PCR amplification of the target locus (see Supplementary Table 3) and restriction digestion with EcoRI, ii) direct sequencing of the PCR product and iii) sequencing of individual TOPO-cloned PCR product. The identification of potential off-target loci (see Supplementary Table 2) was performed using a combination of Bowtie, BEDTools, and custom Python scripts. All 20 mers having less than 4 mismatches and all 12 mers with a perfect match to the Rubicon-E5F2 target sequence that were followed by the PAM sequences 5'-NAG-3' or 5'-NGG-3' were identified from the mouse genome assembly NCBI37/mm9 and were considered as potential off-target loci. Each potential off-target site was amplified using a specific primer pair (see Supplementary Table 3) flanking the potential off-target locus. The PCR products were then sequenced. None of the potential off-target sites were modified by the Rubicon-E5F2-sgRNA/Cas9 complex.

Mice and cell lines

All mice were housed pathogen-free. GFP-LC3^{+} mice were kindly provided by Noboru Mizushima (Tokyo Medical and Dental University). ULK1$^{-/-}$ mice were kindly provided by Mondira Kundu (St. Jude Children's Research Hospital) and were bred to GFP-LC3^{+} mice to generate ULK1$^{-/-}$ GFP-LC3^{+} mice. ATG7$^{flox/flox}$ mice (kindly provided by Masaaki Komatsu at The Tokyo Metropolitan Institute of Medical Science) were bred to LysM-Cre+ mice (kindly provided by Peter Murray, St. Jude Children's Research Hospital) and subsequently bred with GFP-LC3 mice to generate LysM-Cre^{+} or LysM-Cre^{-} ATG7$^{flox/flox}$ GFP-LC3^{+} mice. NOX2$^{-/-}$ mice were purchased from Jackson Laboratories and bred with GFP-LC3^{+} mice to generate NOX2$^{-/-}$ GFP-LC3^{+} mice. LysM-Cre^{+} ATG14$^{flox/flox}$, LysM-Cre^{+} ATG3$^{flox/flox}$, LysM-Cre^{+} ATG12$^{flox/flox}$, LysM-Cre^{+} ATG16L$^{flox/flox}$, ATG4B$^{-/-}$, and ATG16LT316A mice were kindly provided by Herbert W. Virgin (Washington University). LysM-Cre^{+} Beclin1$^{flox/flox}$ (Edmund Rucker, University of Kentucky), LysM-Cre^{+} ATG5$^{flox/flox}$

(Tom Ferguson, Washington University), LysM-Cre^{+} VPS34$^{flox/flox}$ (Richard Flavell, Yale University), and LysM-Cre^{+} FIP200$^{flox/flox}$ (Jun-Lin Guan, University of Michigan) were bred to GFP-LC3^{+} mice to generate GFP-LC3^{+} versions of these strains. Rubicon$^{+/+}$ and Rubicon$^{-/-}$ mice were generated using CRISPR/Cas9 gene editing technology (see above) and were bred to GFP-LC3^{+} mice to generate Rubicon$^{+/+}$ and Rubicon$^{-/-}$ GFP-LC3^{+} mice.

Bone marrow-derived macrophages (BMDMs) were generated from bone marrow progenitors obtained from littermates. Freshly prepared bone marrow cells were cultured in DMEM medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES buffer, 50 µg/ml penicillin, and non-essential amino acids in the presence of 20 ng/ml rmM-CSF (Peprotech) for 6 days. Unattached cells were removed on day 6, and attached macrophages were detached from plates and re-plated for experimental use. RAW-GFP-LC3[57] cells were grown in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 µg/ml penicillin, non-essential amino acids, sodium pyruvate, and 2-mercaptoethanol (55 µM).

Constructs

BMDMs were transfected using Lipofectamine LTX with Plus™ according to manufacturer's protocol. GFP-LC3 construct was kindly provided by Annete Khaled (USF). Construct expressing PX-mCherry (PI(3)P) binding PX-domain of p40-PHOX) has been previously described[57]. Lentiviral Scrambled and shRubicon constructs were a generous gift from Jae Jung (University of Southern California)[58]. RavZ-GFP and RavZΔ-GFP constructs were generous gifts of Craig Roy (Yale University)[59].

Transduction of Lentiviral Scrambled and shRubicon constructs

Lentiviruses were produced by transient transfection using packaging plasmids (psPAX2 and pMD2.VSV-G) after Lipofectamine 2000-mediated transient transfection into 293T cells. Virus-containing media were collected 72 hr. post-transfection and harvested for the viral particles by passing the supernatants through a 0.45 μm filter. The supernatants were used to infect 1×10^5 RAW-GFP-LC3 cells in 4-well chamber slides in the presence of 8 μg/ml Polybrene. After 24 hours, the medium was freshly replaced, and cells were incubated for additional 3 days for Rubicon knockdown.

siRNA gene silencing of UVRAG, Ambra1, WIPI2, and RAB7

Knock down of mouse UVRAG, Ambra1, WIPI2, and RAB7 using siRNA was performed as previously described[60] with ON-TARGET plus siRNA obtained from Dharmacon. RAW-GFP-LC3 cells were transfected with 50 μM of each siRNA with Lipofectamine RNAi Max according to the manufacturer's recommendations (Invitrogen).

Autophagy and LAP Induction

BMDMs and RAW cells were induced to undergo autophagy by 18 hours of rapamycin treatment (200 nM) or 18 hours under starvation conditions (EBSS). Alexa Fluor® 594 labeled zymosan particles (Invitrogen) were added to macrophage cultures at a ratio of 8:1 (particle:cell). Pam3csk4-coupled[57], Catalase-, or Pam3csk4+Catalase-coupled beads were added to macrophage cultures at a ratio of 10:1 (bead:cell). Biotinylated HRP (Invitrogen) was conjugated to Streptavidin beads (Bangs Laboratories).

Reagents

Class III PI3K activity was inhibited by 3-MA treatment (25 mM, 5 mM). ROS was scavenged using Tiron (Sigma, 1 mM, 0.5 mM). ROS was induced with *tert*-butyl hydroperoxide (TBHP, Sigma, 100 μM, 50 μM).

Real-Time RT-PCR

Total RNA was isolated from cells and tissue using NucleoSpin II kit (Macherey-Nagel) according to the manufacturer's instructions. First strand synthesis was performed using M-MLV reverse transcriptase (Invitrogen). Real time PCR™ was performed using SYBR GREEN PCR master mix (Applied Biosystems), in a Applied Biosystems 7900HT thermocycler using SyBr Green detection protocol as outlined by the manufacturer using the following PCR conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. mRNA expression was normalized against actin, allowing comparison of mRNA levels. Primers used as listed in Supplementary Table 4.

Cell lysis and immunoblotting

Cells were lysed in RIPA buffer for 30 min on ice (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% DOC, 0.1% SDS, protease inhibitor tablet [Roche], 1 mM NaF, 1 mM Na_3VO_4, and 1 mM PMSF). After centrifugation (13.2k rpm, 15 minutes, 4°C), supernatants were analyzed by SDS-PAGE. Anti-LC3B (ab48394) antibody was from abCam. Anti-GATE16 (EP4808) antibody was from Origene. Anti-Actin antibody (C4) was from MP Biomedicals. Anti-ATG7, anti-Beclin1, anti-UVRAG, anti-VPS34, anti-Rubicon, anti-p-p40PHOX, anti-UNC93B, anti-ATG14, anti-LC3A (D50G8), and anti-GABARAP (E1J4E) antibodies were from Cell Signaling. p22PHOX antibody was from Santa Cruz Biotechnology. Anti-RAB5 and anti-RAB7 antibodies were from Sigma-Aldrich.

Phagosomes from BMDMs and RAW cells were obtained as previously described[61, 62]. Briefly, after culture of cells with Pam3csk4-coupled beads, the cells were washed in cold PBS, pelleted, resuspended in 1 ml of homogenization buffer (250 mM sucrose, 3

mM imidazole, pH 7.4), and homogenized on ice in a Dounce homogenizer. Phagosomes were then isolated by flotation on a sucrose step gradient during centrifugation for 1 hr. at 100,000 g at 4°C. Latex-bead phagosomal fraction was then collected from the interface of the 10% and 25% sucrose solutions and resuspended in RIPA buffer for protein immunoblot analysis. The entire phagosome purification was run on 1 – 2 SDS-PAGE gels due to the relatively lower protein yield compared to whole-cell lysate samples. Membranes were sectioned according to the molecular weight marker, and proteins residing within that range of molecular weights were probed with antibodies listed above. When necessary, membranes were stripped with Restore PLUS Western Blot Stripping Buffer (Life Technologies), re-blocked in 1X TBST with 5% w/v nonfat dry milk, and probed with fresh antibodies. Images were captured with an Amersham Imager 600 and saved as tiff files.

Time-Lapse Imaging and Microscopy

Cells were plated on fibronectin-coated glass bottom chamber slide (MatTek, Ashland, MA). Confocal microscopy was performed using the following systems:

-Spinning Disk Confocal Microscopy (SDC):

SDC on live cells was performed with a Marianas SDC imaging system (Intelligent Imaging Innovations/3i) consisting of a CSU22 confocal head (Yokogowa Electric Corporation), DPSS lasers (CrystaLaser) with wavelengths of 445nm, 473nm, 523nm, 561nm, and 658 nm, and a Carl Zeiss 200M motorized inverted microscope (Carl Zeiss MicroImaging, Thornwood), equipped with spherical aberration correction optics (3i). Temperature was maintained at ~37°C and 5% CO2 using an environmental control chamber (Solent Scientific, UK). Images were acquired with a Zeiss Plan-Neofluar 40x

1.3 NA DIC objective on a CascadeII 512 EMCCD (Photometrics), using SlideBook 6 software (3i).

-Laser Scanning Confocal Microscopy (LSCM):

LSCM on live cells was performed with a Nikon TE2000-E inverted microscope equipped with a C1Si confocal system, (Nikon), an argon ion laser at 488nm and DPSS lasers at 404nm and 561 nm (Melles Griot). Temperature was maintained at ~37°C and 5% CO2 using an environmental control chamber (InVivo Scientific). Images were taken at the intervals indicated in the figure legends using an oil-immersion Nikon Plan Fluor 40X 1.3 NA objective with phase contrast optics.

Flow cytometry analysis

At the indicated time points, GFP-LC3^{+} cells were harvested, washed once with FACS buffer, and permeabilized with digitonin (Sigma, 200 µg/ml) for 15 minutes on ice. Cells were then washed 3 times with FACS buffer and analyzed by flow cytometry for membrane-bound GFP-LC3-II. Likewise, PX-mCherry^{+} cells were harvested, washed once with FACS buffer, and treated with digitonin (200 µg/ml) for 15 minutes on ice. Cells were then washed 3 times with FACS buffer and analyzed by flow cytometry for membrane-bound PI(3)P. For ROS detection, cells were incubated in the dark with 1 µM dihydroethidium (DHE, Invitrogen) for 30 min at 37 °C. Data were acquired using a FACScan cytometer (BD).

Quantification of Phagocytosis

Phagocytosis was calculated using flow cytometry analysis (described above). Percent phagocytosis equals the number of macrophages that have engulfed Alexa Fluor 594-zymosan or *A. fumigatus*-dsRed. Quantification of the extent of phagocytosis is

representative of the mean fluorescence intensity (MFI) of the engulfed Alexa Fluor 594-zymosan or *A. fumigatus*-dsRed. Calculation is an average of four independent experiments.

Class III PI3K Activity Assay

LAPosomes were purified as previously described[61, 62]. mVPS34 was immunoprecipitated and incubated with phosphatidylinositol (PI). The quenched PI3K reactions are then subjected to a Class III PI3K Activity Assay (Echelon Biosciences), a competitive ELISA in which the signal is inversely proportional to the amount of PI(3)P produced. Reaction products are diluted and added to the PI(3)P-coated microplate, for competitive binding to a PI(3)P detector protein. The amount of PI(3)P detector protein bound to the plate is determined through colorimetric detection.

Immunofluorescence

Cells grown and stimulated in chamber slides were fixed with 4% formaldehyde for 20 minutes at 4^{o}C. Following fixation, cells were blocked and permeabilized in block buffer (1% BSA, 0.1% Triton in PBS) for 1 hour at RT. Cells were incubated overnight at 4oC with primary antibody diluted 1/200 in block buffer. Cells were washed extensively in TBS-Tween (Tris-buffered saline containing 0.05% Tween-20) and incubated Alexa-Fluor conjugated secondary antibodies (Invitrogen). Images were analyzed using an Olympus BX51 FL Microscope and Slidebook software. Alexa Fluor 647-LAMP1 antibody was from eBioscience. Anti-oxLDL antibody was from Bioss Antibodies, and anti-PI(3)P antibody was from Echelon Biosciences. Anti-LC3B (ab48394) antibody was from abCam. Anti-Beclin1, anti-UVRAG, anti-VPS34, anti-Rubicon, anti-p-p40PHOX, and anti-ATG14 antibodies were from Cell Signaling. Anti-ATG7 antibody was from Sigma-Aldrich. p22PHOX antibody was from Santa Cruz Biotechnology

Mouse Macrophage Infection with Aspergillus fumigatus.

Viable conidia (>95%) from the *Aspergillus fumigatus* Af293 strain or *Aspergillus fumigatus*-dsRed (a generous gift from Jean-Paul Latge at Institut Pasteur) were obtained by growth on Sabouraud dextrose agar (Difco Laboratories) at room temperature[63]. BMDM were plated in triplicate at 5 x 10^5 cells/well, and infected with *Aspergillus fumigatus* at an MOI of 1 or 5. Cells were lysed in 0.1% Triton-X at the indicated timepoints, plated on SD medium in serial dilutions, and incubated at 37°C for 18 hours. CFU was enumerated as previously described[63]. Data are presented as mean ± s.d.

Experimental Invasive Pulmonary Aspergilliosis in Mice

Viable conidia (>95%) from the Aspergillus fumigatus Af293 strain were obtained by growth on Sabouraud dextrose agar (Difco Laboratories) at room temperature. For infection, mice were anesthetized with Isofluorane before the intranasal instillation of a suspension of 2 x 10^7 conidia per 20 µL of saline. Mice were monitored for fungal growth (cfu per lung, mean ± SD), cytokine production (serum and lung), and histopathology (H&E and Gomori staining of lung tissue sections). Histology sections were imaged on a NikonE800 microscope, and images were captured using a high-resolution DS-Fi1 camera (Nikon).

Cytokine Detection

Serum was collected from *A. fumigatus*-infected mice at day 7 post-infection. Cytokines were analyzed by Luminex technologies (Millipore).

SILAC labeling of phagosome purification

The SILAC labeling was performed as previously described[64]. Briefly, RAW 264.7 cells were cultured and differentiated in Dulbecco's modified Eagle's medium (deficient in L-Lysine and L-Arginine, Thermo Fisher Scientific) supplemented with 5% dialyzed fetal calf serum (Atlanta Biologicals). L-Proline (Thermo Fisher Scientific) was added at 200 µg/ml to prevent L-Arginine-to-L-Proline conversion. The Lys and Arg amino acids were added in light forms or heavy forms (Lys8: [$^{13}C_6{}^{15}N_2$], Arg10: [$^{13}C_6{}^{15}N_4$], Thermo Fisher Scientific) to a final concentration of 0.26mM. The cells were cultured for at least seven passages for complete labeling. The first experimental pair will consist of "light" cells treated with inert beads (non-LAP) and "heavy" cells treated with Pam3csk4- coupled beads (LAP). The second experimental pair will consist of "light" cells treated with IgG-coupled beads (LAP) and "heavy" cells treated with inert beads (non-LAP). After stimulation, the phagosomes were purified from each sample as described above and lysed in SDS loading buffer. Phagosomal proteins extracted from LAP samples (Pam3csk4- or IgG-coupled beads) were mixed with Non-LAP samples (inert beads) at 1:1 ratio for comparison.

Mass spectrometry analysis

The MS analysis was carried out using our optimized platform[65]. The two mixed SILAC samples were resolved on a 10% SDS-PAGE gel and stained with Coomassie Blue. Each gel lane was cut into 10 gel bands, followed by in-gel trypsin digestion with Cys alkylation. The extracted peptides were loaded onto a C_{18} column (75 µm i.d. x 10 cm, 15 µm orifice and 2.7 µm resin, New Objective), eluted during a 10–35% gradient in 90min (Buffer A: 0.2% formic acid; Buffer B: 0.2% formic acid with 70% acetonitrile, 400 nl/min), and analyzed by MS (LTQ Orbitrap ELITE, Thermo Fisher Scientific). The MS setting included one MS scan (1 x 10^6 automatic gain control, 100 ms maximal ion time, 240K resolution) followed by top 20 data-dependent low resolution MS/MS scans (rCID,

2 *m/z* isolation width, 35 normalized collision energy, 3000 automatic gain control, and 100 ms maximal ion time).

Acquired MS/MS spectra were extracted and searched against Uniprot mouse databases by the Sequest algorithm[66] with the target-decoy strategy[67] to analyze false discovery rate (FDR). Searching parameters included mass tolerance of precursor ions (± 10 ppm) and product ion (±0.5 Da); partially tryptic restriction; fixed modification of carboxamidomethylated Cys (+57.02146 Da); dynamic modifications of oxidized Met (+15.99492 Da), stable isotope labeled Lys (+8.01420 Da) and Arg (+10.00827 Da); three maximal modification sites and 5 maximal missed cleavages. Only *b* and *y* ions were considered during the database match. Matched peptide spectra were first filtered by MS mass accuracy. Then the survived matches were grouped by precursor ion charge state and further filtered by Xcorr and ΔCn values to reduce protein FDR to approximately 1%. The spectra of listed proteins were manually evaluated.

Statistical Analysis. The statistical significance of differences in mean values was calculated using unpaired, two-tailed Student's *t* test. p values less than 0.05 were considered statistically significant.

References

55 Wang, H. *et al.* One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* **153**, 910-918, doi:10.1016/j.cell.2013.04.025 (2013).

56 Pelletier, S., Gingras, S. & Green, D. R. Mouse Genome Engineering via CRISPR-Cas9 for Study of Immune Function. *Immunity* **42**, 18-27, doi:10.1016/j.immuni.2015.01.004 (2015).

57 Sanjuan, M. A. *et al.* Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* **450**, 1253-1257 (2007).

58 Yang, C. S. *et al.* The autophagy regulator Rubicon is a feedback inhibitor of CARD9-mediated host innate immunity. *Cell Host Microbe* **11**, 277-289, doi:10.1016/j.chom.2012.01.019 (2012).

59 Choy, A. *et al.* The Legionella effector RavZ inhibits host autophagy through irreversible Atg8 deconjugation. *Science (New York, N.Y* **338**, 1072-1076, doi:10.1126/science.1227026 (2012).
60 Martinez, J. *et al.* Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 17396-17401, doi:10.1073/pnas.1113421108 (2011).
61 Desjardins, M., Huber, L. A., Parton, R. G. & Griffiths, G. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. *J Cell Biol* **124**, 677-688 (1994).
62 Henault, J. *et al.* Noncanonical autophagy is required for type I interferon secretion in response to DNA-immune complexes. *Immunity* **37**, 986-997, doi:10.1016/j.immuni.2012.09.014 (2012).
63 de Luca, A. *et al.* IL-1 receptor blockade restores autophagy and reduces inflammation in chronic granulomatous disease in mice and in humans. *Proc Natl Acad Sci U S A* **111**, 3526-3531, doi:10.1073/pnas.1322831111 (2014).
64 Ong, S. E. *et al.* Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* **1**, 376-386 (2002).
65 Xu, P., Duong, D. M. & Peng, J. Systematical optimization of reverse-phase chromatography for shotgun proteomics. *Journal of proteome research* **8**, 3944-3950, doi:10.1021/pr900251d (2009).
66 Eng, J. K., McCormack, A. L. & Yates, J. R. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *Journal of the American Society for Mass Spectrometry* **5**, 976-989, doi:10.1016/1044-0305(94)80016-2 (1994).
67 Elias, J. E. & Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nature methods* **4**, 207-214, doi:10.1038/nmeth1019 (2007).