Supplementary Figures and Tables

Figure S1. Sequence analysis of ATG4 cysteine peptidases

A. Amino acid alignment of *L. major* ATG4 aligned with those of *T. brucei*, *S. cerevisiae*, *H. sapiens* and *A. thaliana*. Identical and conserved amino acids are shaded in black and grey, respectively. Gaps are indicated by hyphens and insertions removed from the alignment are indicated in parenthesis indicating the number of residues removed. Similarly, some N-terminal residues are not shown. The catalytic (β1-7) and auxiliary (β8-13) domains (based on Sugawara *et al*., [1]), the sequence linking the two domains, and the inhibitory loop are indicated by single, double and triple lines, respectively. Residues required for substrate recognition and hydrolysis according to Sugawara *et al*. [1] are marked: catalytic triads (∗); oxyanion hole (Δ), substrate recognition (o, Π). Key: Lmj, *L. major*; Tb, *T. brucei*; Sc, *S. cerevisiae*; Hs, *H. sapiens*; At, *A. thaliana*. GenBank accession numbers and GeneDB systematic names are: LmjATG4.1, LmjF32.3890; LmjATG4.2, LmjF30.0270; TbATG4.1, Tb11.01.7970, TbATG4.2, Tb927.6.7690; ScATG4, P53867; HsATG4.1, autophagin-1, CAD43219; HsATG4.2, autophagin-2, CAD43218; HsATG4.3, autophagin-3, Q96DT6; HsATG4, autophagin-4, NP116274; AtATG4a, At2g44140.1; AtATG4b, At3g59950.1.

Figure S2. Analysis of ATG8 proteins.

A. Amino acid alignment of *L. major* ATG8 (i) and ATG12 (ii) proteins aligned with those of *S. serevisiae*, *H. sapiens* and *A. thaliana*. Identical and conserved amino acids are shaded in black and grey, respectively. Gaps are indicated by hyphens. The alpha helices (α) and beta folds (β) of the ubiquitin-like fold conserved in *H. sapiens*

(GABARAP, [2]; GATE-16, [3]; MAP-LC3, [1]) and *A. thaliana* [4]ATG8s are marked. The scissile glycine (*) hydrolysed by ATG4 and residues (#) that interact with the E1-like enzyme ATG7 [1] are marked. Residues that recognise and interact with the active site of the peptidase is marked with ψ. [5] Residues used as the basis for the peptide substrates synthesised are underlined. Key: Lmj, *L. major*; Tb, *T. brucei;* Sc, *S. cerevisiae;* Hs, *H. sapiens;* At, *A. thaliana.* GenBank accession numbers are: Sc, P38182; Hs-GATE-16, 1EO_6_A; Hs-GABARAP, CAG47031; Hs-MAP-LC3A, Q62625; At-ATG8A, NP_567642; At-ATG8E, NP_850431; At-ATG8H, NP_566518. GeneDB systematic names for the *L. major* genes are: LmjF19.1630 (ATG8); LmjF22.1300 (ATG12); LmjF19.0820 (ATG8B.1); LmjF19.0840 (ATG8A.1); LmjF19.0842 (ATG8B.2); LmjF19.0844 (ATG8A.2); LmjF19.0846 (ATG8B.3); LmjF19.0848 (ATG8A.3); LmjF19.0850 (ATG8B.4); LmjF19.0860 (ATG8B.5); LmjF19.0870 (ATG8B.6); LmjF19.0880 (ATG8B.7); LmjF19.0900 (ATG8B.8); LmjF19.0910 (ATG8B.9); Lmj09.0150 (ATG8C.1); Lmj09.0152 (ATG8C.2); Lmj09.0154 (ATG8C.3); Lmj09.0156 (ATG8C.4); Lmj09.0158 (ATG8C.5); Lmj09.0160 (ATG8C.6); Lmj09.0162 (ATG8C.7); Lmj09.0164 (ATG8C.8); Lmj09.0166 (ATG8C.9); Lmj09.0170 (ATG8C.10); Lmj09.0172 (ATG8C.11); Lmj09.0174 (ATG8C.12); Lmj09.0180 (ATG8C.13).

B. Phylogenetic analysis of ATG8 paralogues. Conserved residues pruned of insertions and gaps were used for the analysis. Reliability values after bootstrapping are shown at the nodes. Key: GenBank accession numbers and GeneDB systematic names for *L. major* are as detailed in A. ScATG12, P38316; AtATG12, Q8S924; HsATG12, AAH12266; HIATG12, BAF75797.

A

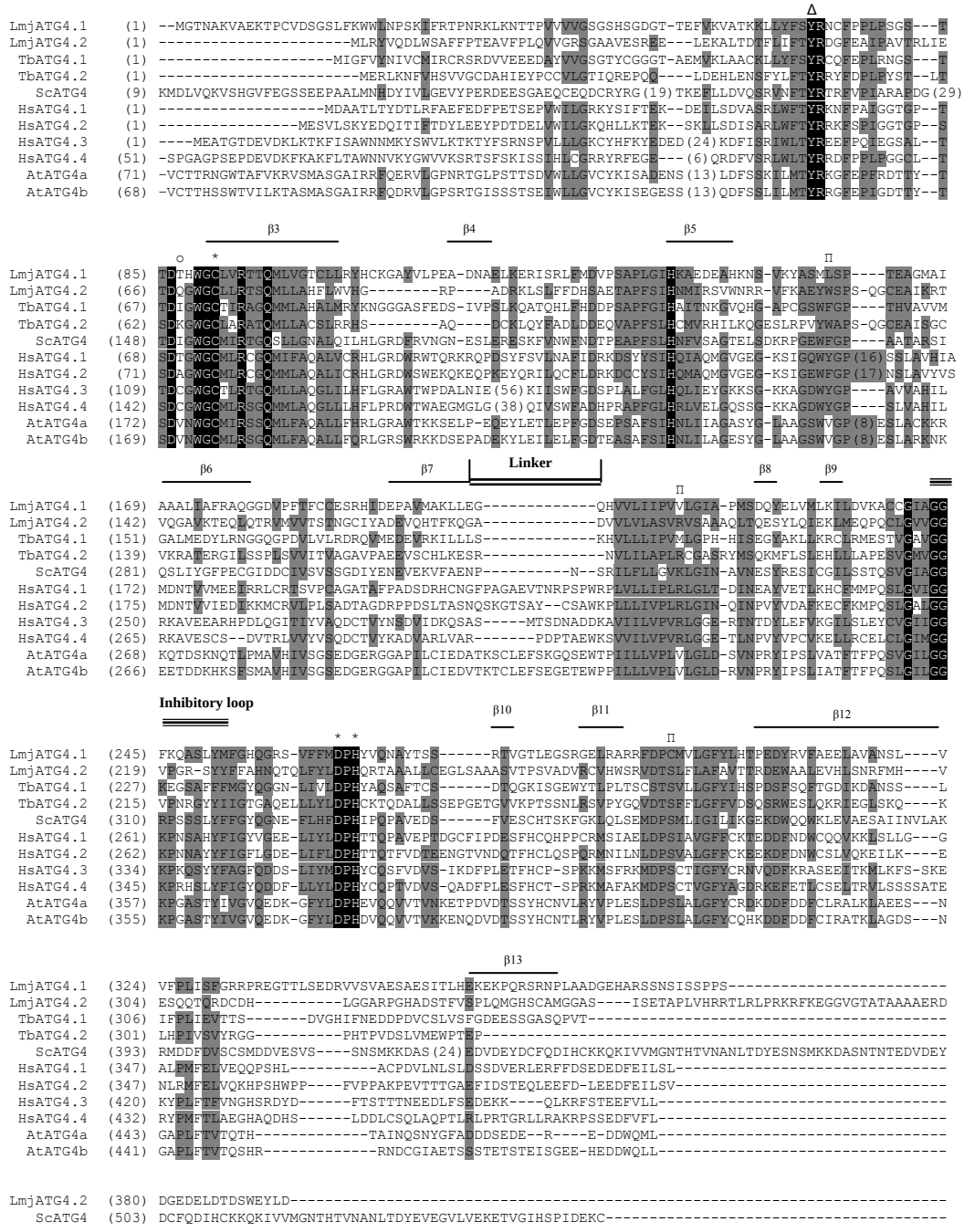
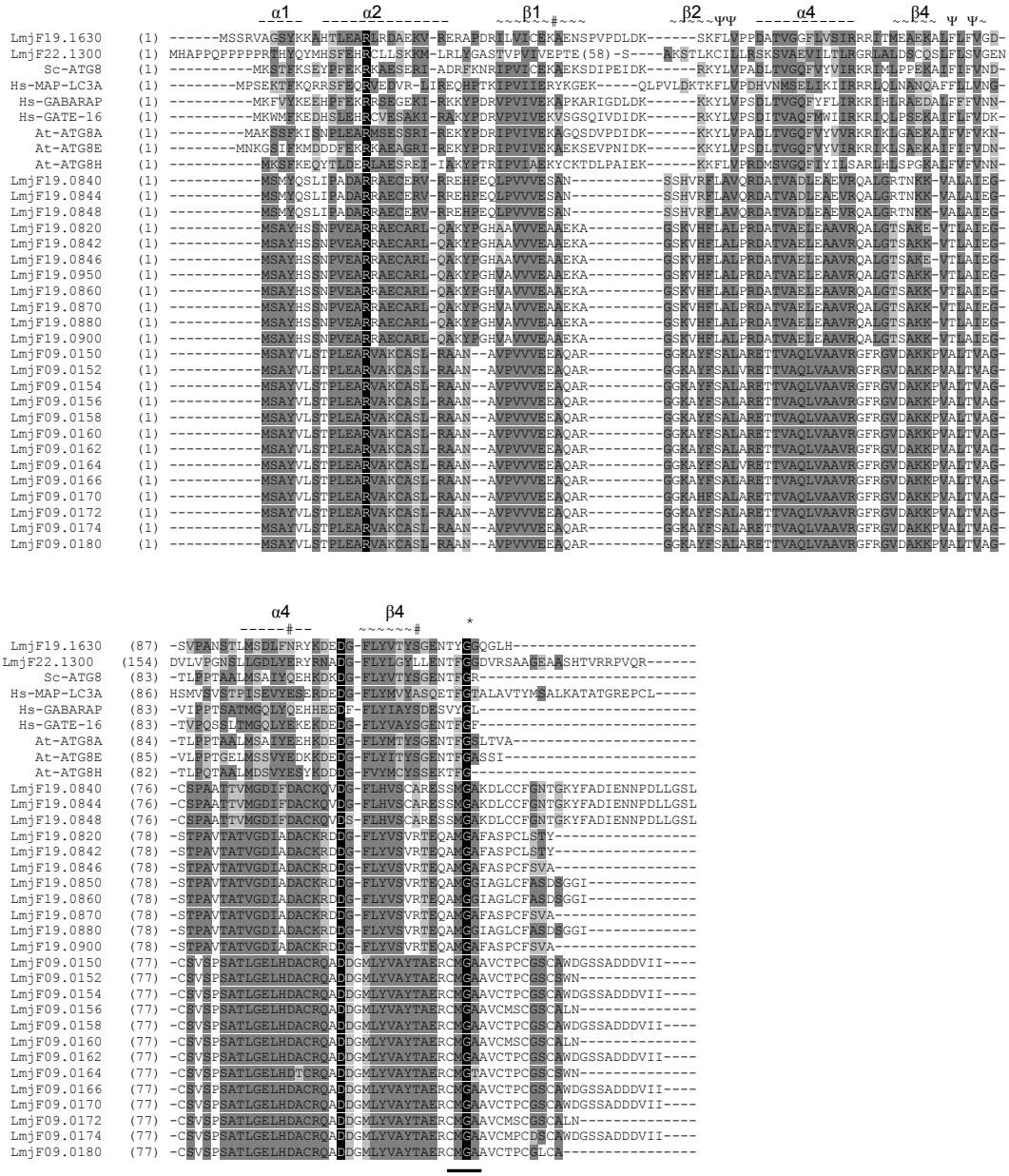

Δ
β3
β4
β5
β6
β7
Linker
β8
β9
Inhibitory loop
β10
β11
β12
β13
LmjATG4.1
LmjATG4.2
TbATG4.1
TbATG4.2
ScATG4
HsATG4.1
HsATG4.2
HsATG4.3
HsATG4.4
AtATG4a
AtATG4b

Figure S1

A

(i)



(ii)

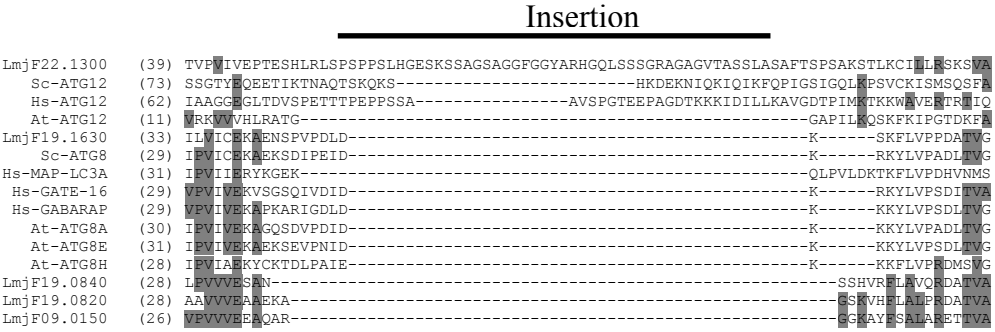


Figure S2

B

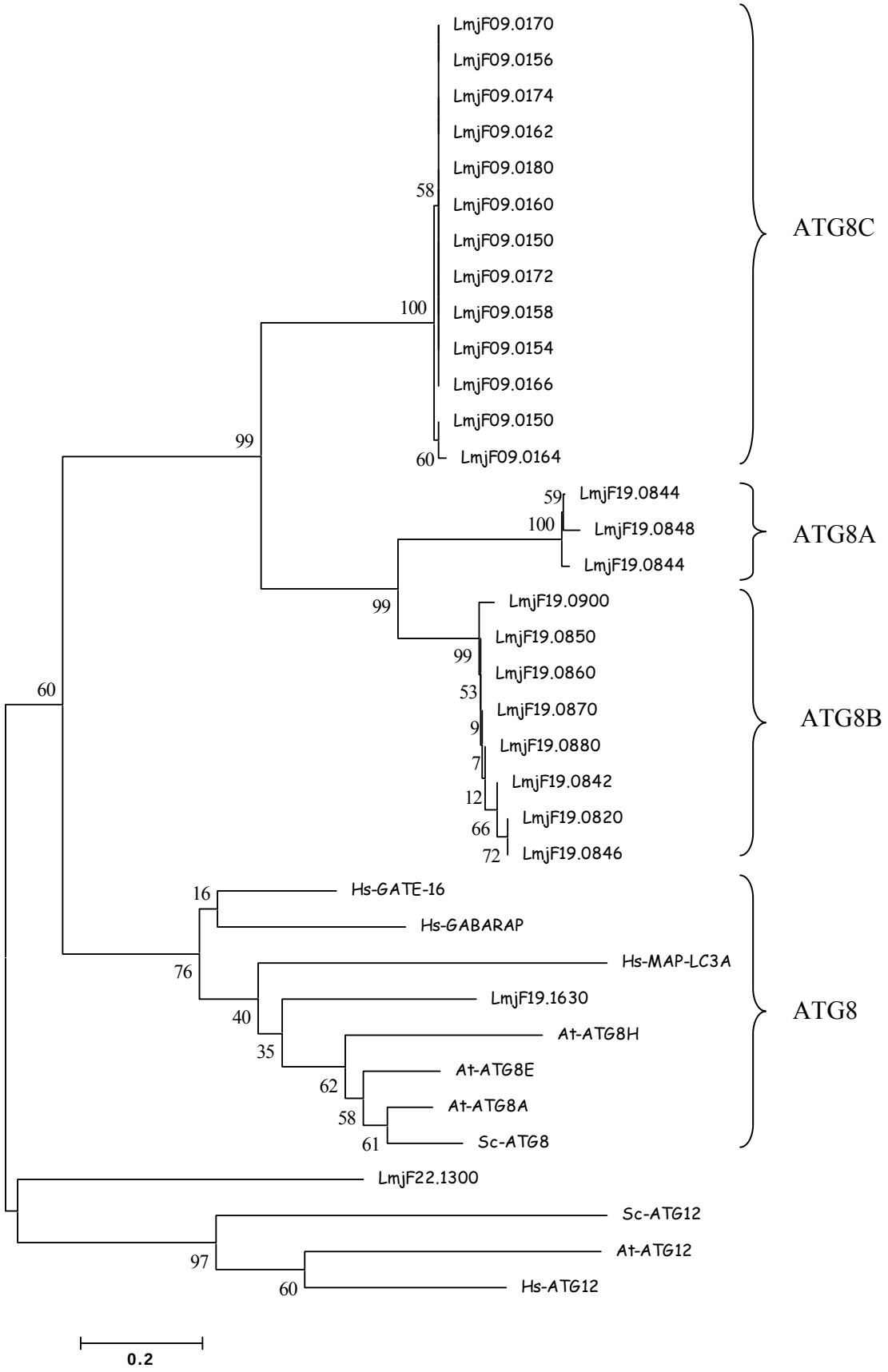

LmjF09.0170
LmjF09.0156
LmjF09.0174
LmjF09.0162
LmjF09.0180
LmjF09.0160
LmjF09.0150
LmjF09.0172
LmjF09.0158
LmjF09.0154
LmjF09.0166
LmjF09.0150
LmjF09.0164
ATG8C
LmjF19.0844
LmjF19.0848
LmjF19.0844
ATG8A
LmjF19.0900
LmjF19.0850
LmjF19.0860
LmjF19.0870
LmjF19.0880
LmjF19.0842
LmjF19.0820
LmjF19.0846
ATG8B
Hs-GATE-16
Hs-GABARAP
Hs-MAP-LC3A
LmjF19.1630
At-ATG8H
At-ATG8E
At-ATG8A
Sc-ATG8
ATG8
LmjF22.1300
Sc-ATG12
At-ATG12
Hs-ATG12
0.2

Figure S2

Table S1. Plasmids used in this study

(i) For transforming *E. coli*

Plasmid	Characteristic	Primer combination
pET21a^{+}ATG4.1	Plasmid containing a 1.1 kb ORF of LmjF32.3890 modified with the *Nde*I/*Xho*1 restriction sites.	NT158 5'CAT ATG GGC ACG AAC GCC AAA GTG GCA GAG3' NT5159 5'CTC GAG GCT CGG TGG AGA AGA GAT TGA ATT CGA GC3'
pET21a^{+}ATG4.2	Plasmid containing a 1.2 kb ORF of LmjF30.0270 modified with the *Nde*I/*Xho*1 restriction sites.	NT160 5'CAT ATG CTC CGC TAC GTG CAA GAT T3' NT161 5'CTC GAG ATC CAG ATA CTC CCA CGA A3'
pET28a^{+}ATG8-HA	Plasmid containing a 0.39 kb ORF of LmjF19.1630 containing the HA tag and modified with the *Nde*I/*Xho*1 restriction sites.	NT208 5'CAT ATG TCT TCC AGA GTA GCT GGG TGG TA3' NT209 5' CTC GAG TCA AGC GTA GTG TGG GAC GTC GTA TGG GTA GTG CAG CCC CTG CCC GCC GTA3'
pET28a^{+}ATG8A-HA	Plasmid containing a 0.42 kb ORF of LmjF19.0840 containing the HA tag and modified with the *Nde*I/*Xho*1 restriction sites.	NT212 5'CAT ATG TCC ATG TAC CAG TCG CTG ATC CCT GCC G3' NT213 5'CTC GAG TCA AGC GTA GTG TGG GAC GTG GTA TGG GTA CAA GGA GCC AAG CAG GTC CGG GT3'
pET28a^{+}ATG8B-HA	Plasmid containing a 0.38 kb ORF of LmjF19.0850 containing the HA tag and modified with the *Nde*I/*Xho*1 restriction sites.	NT210 5' CAT ATG TCC GCC TAC CAC AGC AGC AAC C3' NT211 5' CTC GAG TCA AGC GTA GTG TGG GAC GTC GTA TGG GTA AGC GAC GGA GAA GCA CGG ACT C3'
pET28a^{+}ATG8C-HA	Plasmid containing a 0.42 kb ORF of LmjF09.0150 containing the HA tag and modified with the NdeI/Xho1 restriction sites.	NT390 5'ATC GTA TGG GTA AAT GAT CAC GTC ATC GTC CGC AGA GCT GC3' NT391 5' TGG CAT ATG TCC GCC TAC GTG TTG TCG ACG CCG CTG GAG3'
pET28a^{+}LmjF22.1300-HA	Plasmid containing a 0.63 kb ORF of LmjF22.1300 containing the HA tag and modified with the *Nde*I/*Xho*1 restriction sites.	NT388 5' AAC ATC GTA TGG GTA CCG CTG CAC CGG TCG CCT CAC CGT ATG AG3' NT389 5' TGG CAT ATG CAC GCG CCA CCA CAG CCG CCG CCG CCA CCT CG3'

(ii) For transfecting *L. major*.

Plasmid	Characteristic	Primer combination
pGLATG4.2-HYG5'3' pGLATG4.2-BSD5'3'	Plamid containing the 5' and 3' flanks of *L. major* ATG4.2 gene (LmjF30.0270) for genetic manipulation.	Besteiro *et al.*, 2006
pN-[GFP-ATG8]	Plasmid containing the 0.39 kb ORF of LmjF19.1630 modified with the *Bgl*II/*Xho*I restriction sites for cloning into the pNUS-nHGFP vector.	Besteiro *et al.*, 2006
pN-[GFP-ATG8A]	Plasmid containing the 0.42 kb ORF of LmjF19.0849 modified with the *Bgl*II/*Kpn*I restriction sites for cloning into the pNUS-nGFP vector.	NT250 5'AGA TCT ATG TCC ATG TAC CAG TCG CTG ATC CCT GCC GAC3' NT251 5'GGT ACC CAA GGA GCC AAG CAG GTC CGG GTT ATT CTC3'
pN-[GFP-ATG8B]	Plasmid containing the 0.38 kb ORF of LmjF19.0850 modified with the *Bgl*II/*Kpn*I restriction sites for cloning into the pNUS-nHGFP vector.	NT252 5'AGA TCT ATG TCC GCC TAC CAC AGC AGC AAC CCT GTC GAG GCC C3' NT253 5'GGT ACC AGC GAC GGA GAA GCA CGG ACT CGC AAA GGC3'
pN-[GFP-ATG8C]	Plasmid containing the 0.42 kb ORF of LmjF09.0150 modified with the *Bgl*II/*Xho*I restriction sites for cloning into the pNUS-nHGFP vector.	NT256 5'AGA TCT ATG TCC GCC TAC GTG TTG TCG ACG CCG CTG3' NT257 5'CTC GAG AAT GAT CAC GTC ATC GTC CGC AGA GCT GCC3'
pN[RFP-ATG12]	Plasmid containing the 0.63 kb ORF of LmjF22.1300 modified with the *Bgl*II/*Xho*I restriction sites for cloning into the pNUS-nHRFP vector.	NT350 5'CTC GAG TCA CCG CTG CAC CGG TCG CC3' NT351 5'AGA TCT ATG CAC GCG CCA CCA CAG CC3'
pN-ATG4.2	Plasmid containing the 1.39 kb ORF of LmjF30.0270 modified with the *Nde*I/*Xho*I restriction sites for cloning into the pNUS-nHGFP vector and used to replace GFP in the plasmid.	Besteiro *et al.*, 2006

(iii) For transforming yeast strains.

Plasmid	Characteristic	Primer combination
pCM185[ATG5]	Plasmid containing a 1.38 kb ORF of LmjF30.0980 and modified with the *BamHI*/*Not*1 restriction sites.	NT364 5'GGA TCC ATG CTT CTC GCC ATT GTG CGG GAC CTC CTT3' NT365 5'GCG GCC GCT CAC ACC TGA ACC GTG ACA AAA AT3'
pCM185[ATG10]	Plasmid containing a 0.78 kb ORF of LmjF31.3105 and modified with the *BamHI*/*Not*1 restriction sites.	NT362 5'GGA TCC GAC TGC TCT GAG GCG GTC CTC GTG3' NT363 5'GCG GCC GCT CAT GCA CGT GCG GTA TCG AA3'
pCM185[ATG8]	Plasmid containing a 0.39 kb ORF of LmjF19.1630 (LmjATG8) and modified with the *BamHI*/*Not*1 restriction sites.	NT352 5'GGA TCC ATG TCT TCC AGA GTA GCT GGG TCG TAC AAG3' NT353 5'GCG GCC GCC TAG TGC AGC CCC TGC CC3'
pCM185[ATG8g]	Plasmid containing a 0.39 kb ORF of LmjF19.1630 (LmjATG8) terminating at the scissile glycine and modified with the *BamHI*/*Not*1 restriction sites.	NT352 5'GGA TCC ATG TCT TCC AGA GTA GCT GGG TCG TAC AAG3' NT357 5'GCG GCC GCC TAG TGC AGC CCC TGC CC3'
pCM185[ATG8A]	Plasmid containing a 0.42 kb ORF of LmjF19.0840 (LmjATG8A.1) and modified with the *BamHI*/*Not*1 restriction sites.	NT354 5'GGA TCC ATG TCC ATG TAC CAG TGC CTG ATC CCT GCC GAC3' NT355 5'GCG GCC GCT TAC AAG GAG CCA AGC AGG TCC GGG TTA TTC TC3'
pCM185[ATG8B]	Plasmid containing a 0.38 kb ORF of LmjF19.0850 (LmjATG8B.4) and modified with the *BamHI*/*Not*1 restriction sites.	NT356 5'GGAT CCA TGT CCG CCT ACC ACA GCA GCA ACC CTG TCG AGG CCC3' NT367 5'GCG GCC GCC TAA GCG ACG GAG AAG CAC GGA CTC GCA AAG GC3'
pCM185[ATG8C]	Plasmid containing a 0.42 kb ORF of LmjF09.0150 (LmjATG8C.1) and modified with the *BamHI*/*Not*1 restriction sites.	NT358 5'GGA TCC ATG TCC GCC TAC GTG TTG TCG ACG CCG CTG3' NT359 5'GCG GCC GCT TAA ATG ATC ACG TCA TCG TCC GCA GAG CTG CC3'
pCM185[ATG12]	Plasmid containing a 0.63 kb ORF of LmjF22.1300 and modified with the *BamHI*/*Not*1 restriction sites.	NT360 5'GGA TCC ATG CAC GCG CCA CCA CAG CC3' NT361 5'GCG GCC GCT CAC CGC TGC ACC GGT CGC C3'
pCM185[ATG12g]	Plasmid containing a 0.63 kb ORF of LmjF22.1300 terminating at the scissile glycine and modified with the *BamHI*/*Not*1 restriction sites.	NT360 5'GGA TCC ATG CAC GCG CCA CCA CAG CC3' NT368 5' GCG GCC TCC GCC GAA GGT GTT TTC CAG CAA GTA3'

* pCM vectors were obtained from EUROSCARF
* described previously in Besteiro *et al*., 2006

Table S2. Strains used in this study

(i) *L. major*

Strain	Genotype and characteristics	Reference
Δ*atg4.2*	*L. major* deficient in the cysteine peptidase ATG4.2	Besteiro *et al*., 2006
Δ*atg4.2*[pN-GFP-ATG8]	*L. major* Δ*atg4.2* transfected with pN- GFP-ATG8	Besteiro *et al*., 2006
Δ*atg4.2*[pN-GFP-ATG8A]	*L. major* Δ*atg4.2* transfected with pN-GFP-ATG8A	This study
Δ*atg4.2*[pN-GFP-ATG8B]	*L. major* Δ*atg4.2* transfected with pN-GFP-ATG8B	This study
Δ*atg4.2*[pN-GFP-ATG8C]	*L. major* Δ*atg4.2* transfected with pN-GFP-ATG8C	This study
WT[pN-GFP-ATG8]	*L. major* transfected with pN-GFP-ATG8	Besteiro *et al*., 2006
WT[pN-GFP-ATG8A]	*L. major* transfected with pN-GFP-ATG8A	This study
WT[pN-GFP-ATG8B]	*L. major* transfected with pN-GFP-ATG8B	This study
WT[pN-GFP-ATG8C]	*L. major* transfected with pN-GFP-ATG8C	This study
WT[pN-ATG4.2/pN-GFP-ATG8]	*L. major* co-transfected with pN-GFP-ATG8 and pN-ATG4.2	This study
WT[pN-GFP-ATG8/pN-RFP-ATG12]	*L. major* co-transfected with pN-GFP-ATG8 and pN-RFP-ATG12	This study

(ii) *E. coli*

Strain	Genotype and characteristics	Reference
BL21-ATG4.1-His	BL21(DE3) transformed with pET21a^{+}ATG4.1	This study
BL21-ATG4.2-His	BL21(DE3) transformed with pET21a^{+}ATG4.2	This study
BL21-His-ATG8-HA	BL21(DE3) transformed with pET28a^{+}His-ATG8-HA	This study
BL21-His-ATG8A-HA	BL21(DE3) transformed with pET28a^{+}ATG8A-HA	This study
BL21-His-ATG8B-HA	BL21(DE3) transformed with pET28a^{+}ATG8B-HA	This study
BL21-His-ATG8C-HA	BL21(DE3) transformed with pET28a^{+}ATG8C-HA	This study
BL21-His-ATG8-HA[ATG4.1-His]	BL21(DE3) co-transformed with pET28a^{+}ATG8-HA and pET21a^{+}ATG4.1	This study
BL21-His-ATG8-HA[ATG4.2-His]	BL21(DE3) co-transformed with pET28a^{+}ATG8-HA and pET21a^{+}ATG4.2	This study
BL21-His-ATG8A-HA[ATG4.1-His]	BL21(DE3) co-transformed with pET28a^{+}ATG8A-HA and pET21a^{+}ATG4.1	This study
BL21-His-ATG8A-HA[ATG4.2-His]	BL21(DE3) co-transformed with pET28a^{+}ATG8A-HA and pET21a^{+}ATG4.2	This study
BL21-His-ATG8B-HA[ATG4.1-His]	BL21(DE3) co-transformed with pET28a^{+}ATG8B-HA and pET21a^{+}ATG4.1	This study
BL21-His-ATG8B-HA[ATG4.2-His]	BL21(DE3) co-transformed with pET28a^{+}ATG8B-HA and pET21a^{+}ATG4.2	This study
BL21-His-ATG8C-HA[ATG4.1-His]	BL21(DE3) co-transformed with pET28a^{+}ATG8C-HA and pET21a^{+}ATG4.1	This study
BL21-His-ATG8C-HA[ATG4.2-His]	BL21(DE3) co-transformed with pET28a^{+}ATG8C-HA and pET21a^{+}ATG4.2	This study

(iii) Yeast stains obtained from EUROSCARF

Name	Strain	Genotype
YPL149w	*atg5*Δ	BY4741; Mat a; his3Δ0; leuΔ0; met15Δ0; ura3Δ0; YPL149w::KANmx4
YBL078c	*atg8*Δ	BY4741; Mat a; his3Δ0; leuΔ0; met15Δ0; ura3Δ0; YBL078C::KANmx4
YLL047c	*atg10*Δ	BY4741; Mat a; his3Δ0; leuΔ0; met15Δ0; ura3Δ0; YLL047c::KANmx4
YBR217c	*atg12*Δ	BY4741; Mat a; his3Δ0; leuΔ0; met15Δ0; ura3Δ0; YBR217c::KANmx4
Y00000	Wild type	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0

(iv) Yeast strains generated for this study

Strain	Genotype and characteristics	Reference
*atg5*Δ [pCM185-ATG5]	*atg5*Δ transformed with pCM185- LmjATG5 [LmjF30.0980]	This study
*atg10*Δ [pCM-ATG10]	*atg10*Δ transformed with pCM185-LmjATG10 [LmjF31.3105]	This study
*atg8*Δ [pCM185-ATG8]	*atg8*Δ transformed with pCM185-ATG8 [LmjF19.1630]	This study
*atg8*Δ [pCM185-ATG8g]	*atg8*Δ transformed with pCM185-ATG8 [LmjF19.1630 terminating at the scissile glycine residue]	This study
*atg8*Δ [pCM185-ATG8A]	*atg8*Δ transformed with pCM185-ATG8 [LmjF19.0840]	This study
*atg8*Δ [pCM185-ATG8B]	*atg8*Δ transformed with pCM185-ATG8 [LmjF19.0850]	This study
*atg8*Δ [pCM185-ATG8C]	*atg8*Δ transformed with pCM185-ATG8 [LmjF09.0150]	This study
*atg8*Δ [pCM185-ATG12]	*atg8*Δ transformed with pCM185-ATG12 [LmjF22.1300]	This study
*atg8*Δ [pCM185-ATG12g]	*atg8*Δ transformed with pCM185-ATG12 [LmjF22.1300 terminating at the scissile glycine residue]	This study
*atg12*Δ [pCM185-ATG8]	*atg12*Δ transformed with pCM185-ATG8 [LmjF19.1630]	This study
*atg12*Δ [pCM185-ATG8g]	*atg12*Δ transformed with pCM185-ATG8 [LmjF19.1630 terminating at the scissile glycine residue]	This study
*atg12*Δ [pCM185-ATG8A]	*atg12*Δ transformed with pCM185-ATG8 [LmjF19.0840]	This study
*atg12*Δ [pCM185-ATG8B]	*atg12*Δ transformed with pCM185-ATG8 [LmjF19.0850]	This study
*atg12*Δ [pCM185-ATG8C]	*atg12*Δ transformed with pCM185-ATG8 [LmjF09.0150]	This study
*atg12*Δ [pCM185-ATG12]	*atg12*Δ transformed with pCM185-ATG12 [LmjF22.1300]	This study
*atg12*Δ [pCM185-ATG12g]	*atg12*Δ transformed with pCM185-ATG12 [LmjF22.1300 terminating at the scissile glycine residue]	This study

Sequences alignments and phylogenetic analyses

Amino acid sequences from the ATG8 genes were aligned using the Clustal W algorithm with AlignX program included in the Vector NTI 10.1 package (http://www.invitrogen.com). The data set were converted to a multiple sequence file (MSF) format and the MEGA2 software [6] used to generate a phylogenetic tree using the Neighbor Joining method and Poisson-corrected amino acid distance. The reliability of clustering patterns in the tree was tested by bootstrapping (100 pseudoreplicates).

References

1. Sugawara K, Suzuki NN, Fujioka Y, Mizushima N, Ohsumi Y, Inagaki F. Structural basis for the specificity and catalysis of human Atg4B responsible for mammalian autophagy. J Biol Chem 2005; 280:40058-65

2. Bavro VN, Sola M, Bracher A, Kneussel M, Betz H, Weissenhorn W. Crystal structure of the GABA(A)-receptor-associated protein, GABARAP. EMBO Rep 2002; 3:183-9

3. Paz Y, Elazar Z, FassD. Structure of GATE-16, membrane transport modulator and mammalian ortholog of autophagocytosis factor Aut7p. J Biol Chem 2000; 275:25445-50

4. Chae YK, Im H, Zhao Q, Doelling JH, Vierstra RD, Markley JL. Prevention of aggregation after refolding by balanced stabilization-destabilization: production of the *Arabidopsis thaliana* protein APG8a (At4g21980) for NMR structure determination. Protein Expr Purif 2004; 34:280-3

5. Fass E, Amar N, Elazar Z. Identification of essential residues for the C-terminal cleavage of the mammalian LC3: a lesson from yeast Atg8. Autophagy 2007; 3:48-50

6. Kumar S, Tamura K, Jakobsen IB, Nei M. (2001). MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 2001; 17:1244-45