Supporting Information

LC3B binds to the autophagy protease ATG4b with high affinity using a bipartite interface

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2. Supplementary sequences

1. Supplementary figures and tables
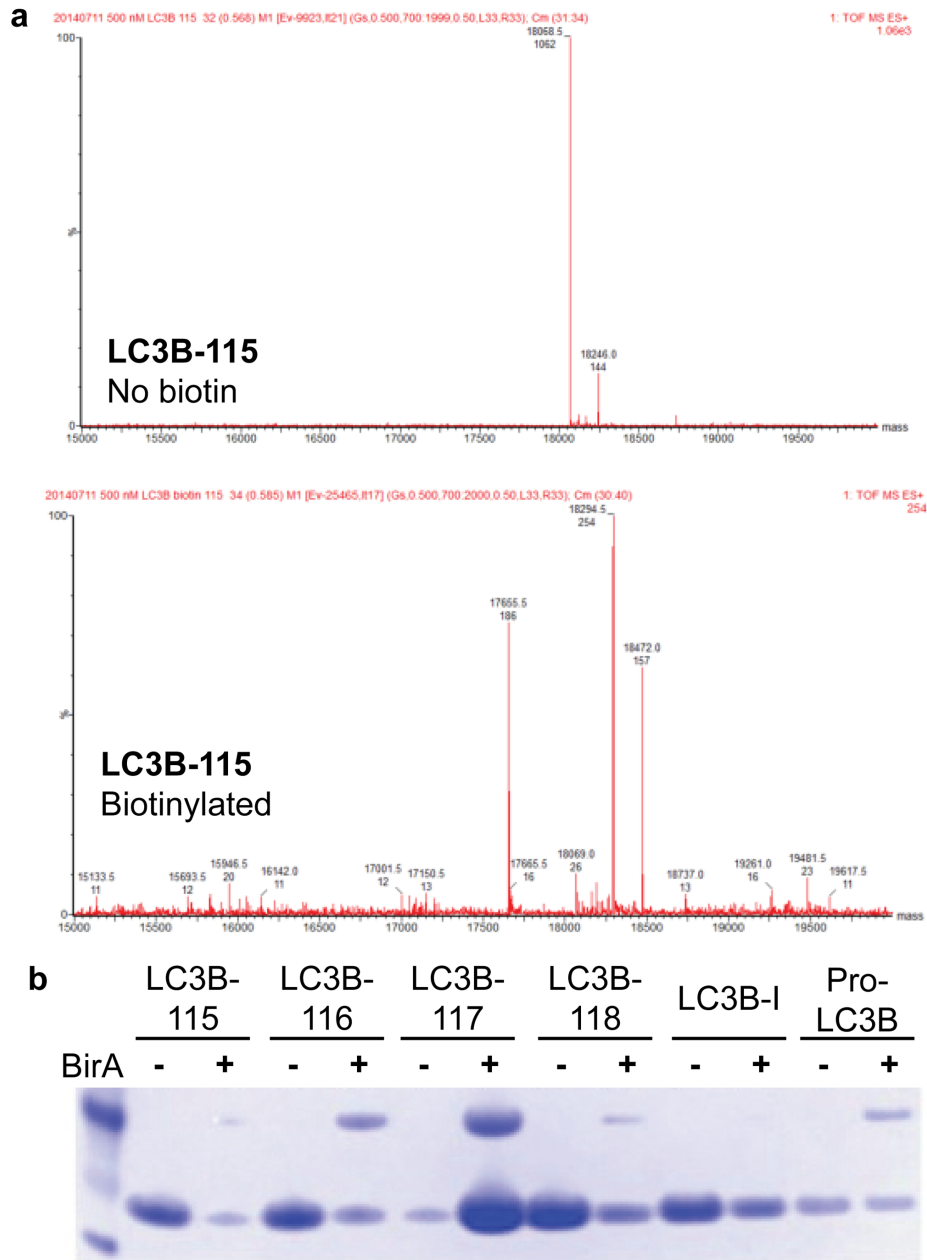
Figure S1. (a) Representative mass spectra for LC3B constructs. The +178 peak is the a-N-gluconoylated version, a common post translational modification observed with proteins expressed with N-terminal His6 tag in *E. coli*. (b) SDS-PAGE protein gel of LC3B proteins.
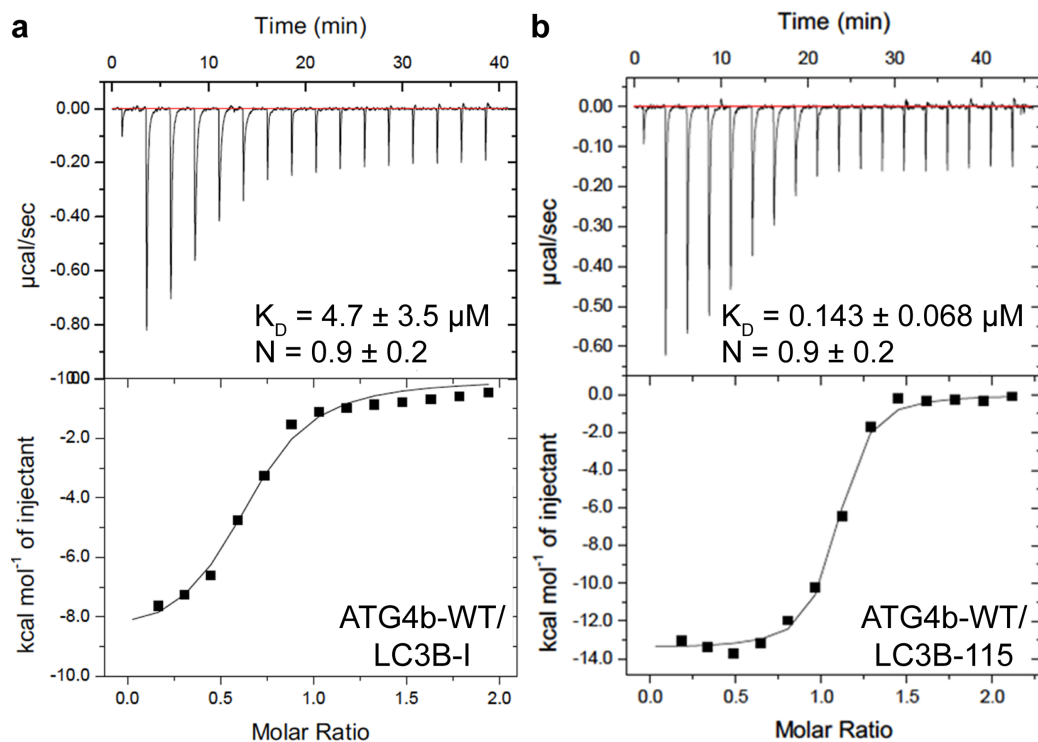
Figure S2. Evaluation of binding between LC3B truncations and ATG4b by isothermal calorimetry (ITC). Representative data are shown for ATG4b WT binding to LC3B-I and LC3B-115: (a) A 700-μM solution of ATG4b was titrated into 68 μM LC3B-I; (b) 100 μM solution of ATG4b titrated into 10 μM LC3B-115. N and K_D values are the mean and standard deviation for five independent experiments; data are shown for one representative measurement.
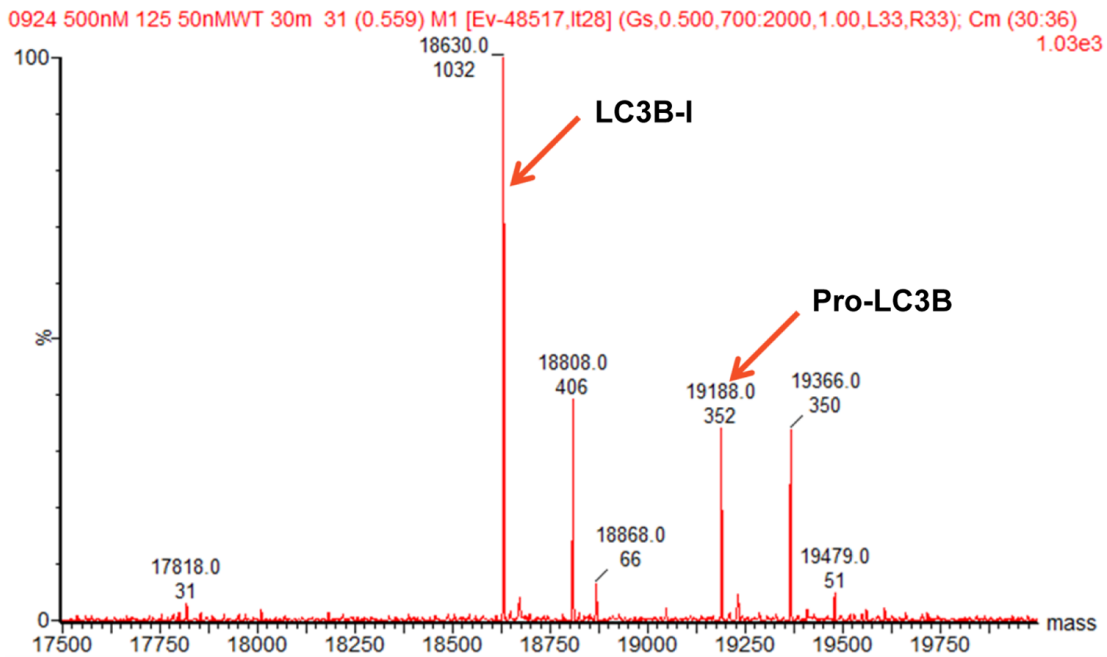
Figure S3. A Representative mass spectrum for an MS-based assay to evaluate the cleavage of pro-LC3B. The arrows are pointing to the masses corresponding to pro-LC3B (substrate) and LC3B-I (product), respectively.
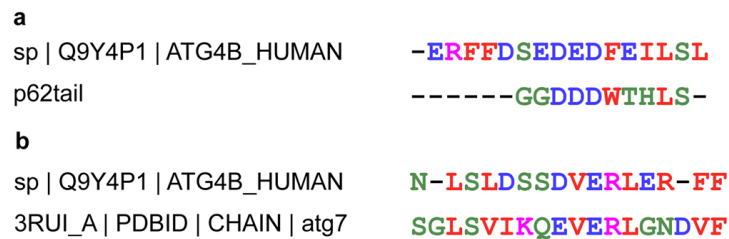
Figure S4. Sequence alignment of the C-terminal tail of ATG4b with (a) p62 tail or (b) ATG7.

Figure S5. SDS-PAGE protein gel for all ATG4b constructs.

Figure S6. Representative SPR sensorgrams of ATG4b proteins binding to LC3B-I fit to a 1:1 kinetic binding model.

Figure S7. Representative SPR sensorgrams of ATG4b proteins binding to LC3B-115 fit to a 1:1 kinetic binding model.

Table S1. Expected masses and observed masses for LC3B constructs.

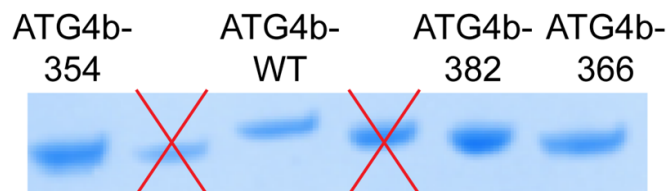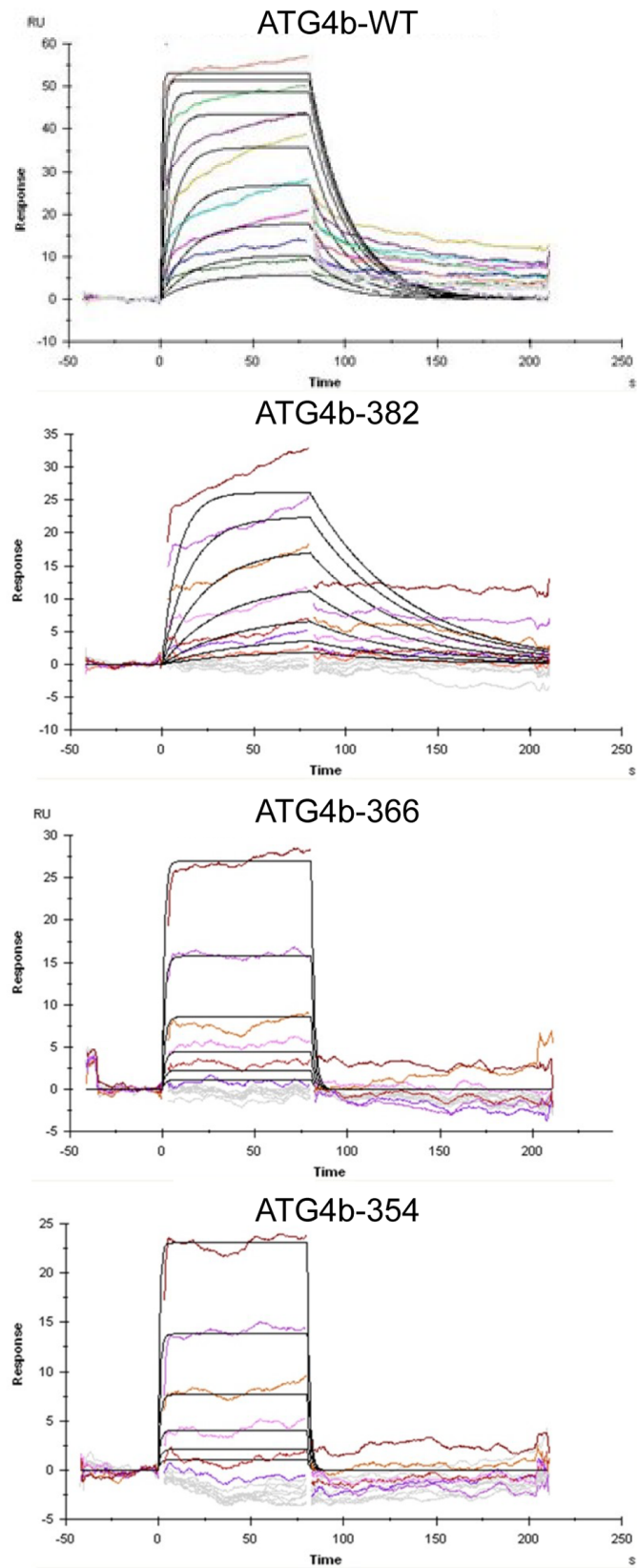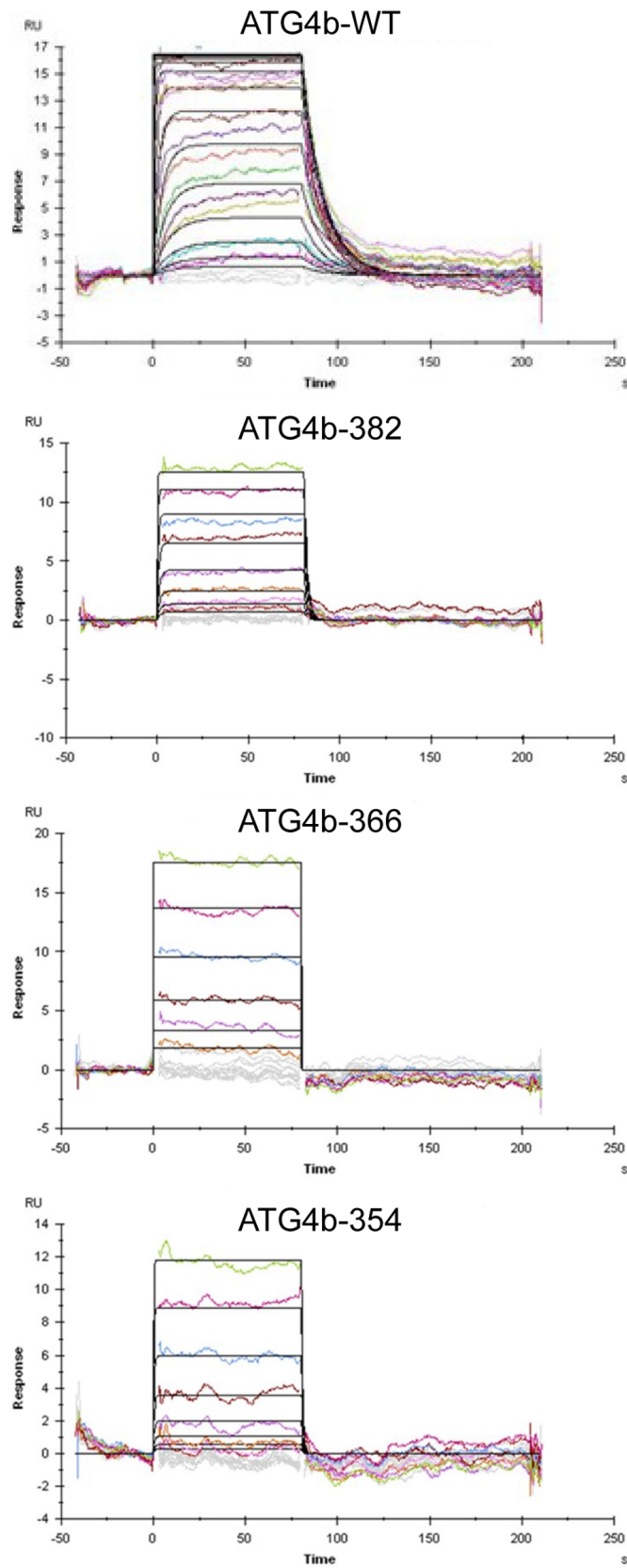|	Expected	Observed	*in vivo* biotinylation (%)
LC3B-115	18068.5	18068.5	91%
Biotinylated LC3B-115	18295.5	18294.5	
LC3B-116	18196.6	18196.5	75%
Biotinylated LC3B-116	18423.6	18423.0	
LC3B-118	18426.8	18426.5	50%
Biotinylated LC3B-118	18653.8	18625.5	
LC3B-I	18631.0	18631.0	27%
Biotinylated LC3B-I	18858.0	18858.0	
Pro-LC3B	19189.8	19189.5	64%
Biotinylated pro-LC3B	19416.0	19416.5	

Table S2. Kinetic rate constants for binding of LC3B-I and LC3B-115 to ATG4b truncations.

	LC3B-I			LC3B-115		
	k_{on} ($M^{-1}S^{-1}$)	k_{off} (S^{-1})	Kinetic K_D (μM)	k_{on} ($M^{-1}S^{-1}$)	k_{off} (S^{-1})	Kinetic K_D (μM)
ATG4b-WT	$8.6 \times 10^5 \pm 9.7 \times 10^4$	0.046 ± 0.02	0.084 ± 0.047	$5.5 \times 10^6 \pm 8.4 \times 10^5$	0.045 ± 0.007	0.0084 ± 0.0019
ATG4b-382	No fit	No fit	No fit	$1.9 \times 10^5 \pm 6.5 \times 10^4$	0.82 ± 0.28	4.4 ± 0.6
ATG4b-366	$1.7 \times 10^4 \pm 1.7 \times 10^4$	0.59 ± 0.22	87 ± 11	$1.4 \times 10^5 \pm 3.8 \times 10^4$	1.3 ± 0.5	9.5 ± 1.4
ATG4b-354	$3.6 \times 10^3 \pm 9.7 \times 10^4$	0.51 ± 0.27	140 ± 74	$7.5 \times 10^4 \pm 3.8 \times 10^4$	1.1 ± 0.4	15.7 ± 8.4

Table S3. Primers used to make ATG4b constructs.

Primer Name	Primer sequences (5'-3')
P1	GCTCGGCGCGCCTGCAGGTCGACATGGACGCAGCTACTCTGACCTAC
P2	GGTTTCTTTACCAGACTCGAGTCAAAGGGACAGGATTTCAAAGTCTTC
P3	GGTTTCTTTACCAGACTCGAGTCACTGCTCCACCAGCTCAAACATGGG
P4	GGTTTCTTTACCAGACTCGAGTCAGTCGAAGAATCTTTCCAGTCGCTC
P5	GGTTTCTTTACCAGACTCGAGTCAGTTCAGGACGTCGGGGCAGGCCAG

Table S4. Primer combinations for ATG4b pET-15b vector constructs.

No.	Primer combination	Constructs	Notes
1	P1 + P2	ATG4b-WT	Full length
2	P1 + P3	ATG4b-354	C-terminal truncation
3	P1 + P4	ATG4b-382	c-terminal truncation
4	P1 + P5	ATG4b-366	c-terminal truncation

Table S5. Primers used to make LC3B constructs.

Primer Name	Primer sequence (5'-3')
P6	ATGCCGTCGGAGAAGACCTTCAAG
P7	CACTGACAATTTCATCCCGAACGTC
P8	CCCGAACGTCTCCTGGGAGGCACAG
P9	CGTCTCCTGGGAGGCACAGACCATG
P10	GGAGGCACAGACCATGTACAGGAATC

Table S6. Primer combinations for LC3B N-His-Avi vector constructs.

No.	Primer combination	Constructs	Notes
1	P6 + P7	Pro-LC3B	Pro-LC3B full length
2	P6 + P8	LC3B-I	LC3B-I full length
3	P6 + P9	LC3B-118	Ubiquitin core + 3 residues
4	P6 + P10	LC3B-115	Ubiquitin core

2. Supplementary sequences

LC3B-115: HisTag—TEV cleavage sequence—AviTag—LC3B(1-115) (Vector backbone: pET-15b)

```
MGSSHHHHHHSSGENLYFQGHMSGLNDIFEAQKIEWHEGGGSMPSEKTFKQRRTFEQRVEDVRLIREQH
PTKIPVIIERYKGEKQLPVLDKTKFLVPDHVNMSELIKIIRRRLQLNANQAFFLLVNGHSMVSVSTPIS
EVYESEKDEDGFLYMVCAS*
```

LC3B-116: HisTag—TEV cleavage sequence—AviTag—LC3B(1-116) (Vector backbone: pET-15b)

```
MGSSHHHHHHSSGENLYFQGHMSGLNDIFEAQKIEWHEGGGSMPSEKTFKQRRTFEQRVEDVRLIREQH
PTKIPVIIERYKGEKQLPVLDKTKFLVPDHVNMSELIKIIRRRLQLNANQAFFLLVNGHSMVSVSTPIS
EVYESEKDEDGFLYMVCASQ*
```

LC3B-118: HisTag—TEV cleavage sequence—AviTag—LC3B(1-118) (Vector backbone: pET-15b)

```
MGSSHHHHHHSSGENLYFQGHMSGLNDIFEAQKIEWHEGGGSMPSEKTFKQRRTFEQRVEDVRLIREQH
PTKIPVIIERYKGEKQLPVLDKTKFLVPDHVNMSELIKIIRRRLQLNANQAFFLLVNGHSMVSVSTPIS
EVYESEKDEDGFLYMVCASQET*
```

LC3B-I: HisTag—TEV cleavage sequence—AviTag—LC3B(1-120) (Vector backbone: pET-15b)

```
MGSSHHHHHHSSGENLYFQGHMSGLNDIFEAQKIEWHEGGGSMPSEKTFKQRRTFEQRVEDVRLIREQH
PTKIPVIIERYKGEKQLPVLDKTKFLVPDHVNMSELIKIIRRRLQLNANQAFFLLVNGHSMVSVSTPIS
EVYESEKDEDGFLYMVCASQETFG*
```

Pro-LC3B: HisTag—TEV cleavage sequence—AviTag—LC3B(1-125) (Vector backbone: pET-15b)

```
MGSSHHHHHHSSGENLYFQGHMSGLNDIFEAQKIEWHEGGGSMPSEKTFKQRRTFEQRVEDVRLIREQH
PTKIPVIIERYKGEKQLPVLDKTKFLVPDHVNMSELIKIIRRRLQLNANQAFFLLVNGHSMVSVSTPIS
EVYESEKDEDGFLYMVCASQETFGMKLSV*
```

ATG4b-WT: HisTag—TEV cleavage sequence—AviTag—AT4b(1-393) (Vector backbone: pET-15b)

```
MGSSHHHHHHSSGENLYFQGHMSGLNDIFEAQKIEWHEGGGSMDAATLTYDTLRFAEFEDFPETSEPVW
ILGRKYSIFTEKDEILSDVASRLWFTYRKNFPAIGGTGPTSDTGWGCMLRCGQMIFAQALVCRHLGRDW
RWTQRKRQPDSYFSVLNAFIDRKDSYYSIHQIAQMGVGEGKSIGQWYGPNTVAQVLKKLAVFDTWSSLA
VHIAMDNTVVMEEIRRLCRTSVPCAGATAFPADSDRHCNGFPAGAEVTNRPSPWRPLVLLIPLRLGLTD
INEAYVETLKHCFMMPQSLGVIGGKPNSAHYFIGYVGEELIYLDPHTTQPAVEPTDGCFIPDESFHCQH
PPCRMSIAELDPSIAVGFFCKTEDDFNDWCQQVKKLSLLGGALPMFELVEQQPSHLACPDVLNLSLDSS
DVERLERFFDSEDEDFEILSL*
```

ATG4b-382: HisTag—TEV cleavage sequence—AviTag—AT4b(1-382) (Vector backbone: pET-15b)

MGSSHHHHHHSSGENLYFQGHMSGLNDIFEAQKIEWHEGGGSMDAATLTYDTLRFAEFEDFPETSEPVW
ILGRKYSIFTEKDEILSDVASRLWFTYRKNFPAIGGTGPTSDTGWGCMLRCGQMIFAQALVCRHLGRDW
RWTQRKRQPDSYFSVLNAFIDRKDSYYSIHQIAQMGVGEGKSIGQWYGPNTVAQVLKKLAVFDTWSSLA
VHIAMDNTVVMEEIRRLCRTSVPCAGATAFPADSDRHCNGFPAGAEVTNRPSPWRPLVLLIPLRLGLTD
INEAYVETLKHCFMMPQSLGVIGGKPNSAHYFIGYVGEELIYLDPHTTQPAVEPTDGCFIPDESFHCQH
PPCRMSIAELDPSIAVGFFCKTEDDFNDWCQQVKKLSLLGGALPMFELVEQQPSHLACPDVLNLSLDSS
DVERLERFFD*

ATG4b-366: HisTag—TEV cleavage sequence—AviTag—AT4b(1-366) (Vector backbone: pET-15b)

MGSSHHHHHHSSGENLYFQGHMSGLNDIFEAQKIEWHEGGGSMDAATLTYDTLRFAEFEDFPETSEPVW
ILGRKYSIFTEKDEILSDVASRLWFTYRKNFPAIGGTGPTSDTGWGCMLRCGQMIFAQALVCRHLGRDW
RWTQRKRQPDSYFSVLNAFIDRKDSYYSIHQIAQMGVGEGKSIGQWYGPNTVAQVLKKLAVFDTWSSLA
VHIAMDNTVVMEEIRRLCRTSVPCAGATAFPADSDRHCNGFPAGAEVTNRPSPWRPLVLLIPLRLGLTD
INEAYVETLKHCFMMPQSLGVIGGKPNSAHYFIGYVGEELIYLDPHTTQPAVEPTDGCFIPDESFHCQH
PPCRMSIAELDPSIAVGFFCKTEDDFNDWCQQVKKLSLLGGALPMFELVEQQPSHLACPDVLN*

ATG4b-354: HisTag—TEV cleavage sequence—AviTag—AT4b(1-354) (Vector backbone: pET-15b)

MGSSHHHHHHSSGENLYFQGHMSGLNDIFEAQKIEWHEGGGSMDAATLTYDTLRFAEFEDFPETSEPVW
ILGRKYSIFTEKDEILSDVASRLWFTYRKNFPAIGGTGPTSDTGWGCMLRCGQMIFAQALVCRHLGRDW
RWTQRKRQPDSYFSVLNAFIDRKDSYYSIHQIAQMGVGEGKSIGQWYGPNTVAQVLKKLAVFDTWSSLA
VHIAMDNTVVMEEIRRLCRTSVPCAGATAFPADSDRHCNGFPAGAEVTNRPSPWRPLVLLIPLRLGLTD
INEAYVETLKHCFMMPQSLGVIGGKPNSAHYFIGYVGEELIYLDPHTTQPAVEPTDGCFIPDESFHCQH
PPCRMSIAELDPSIAVGFFCKTEDDFNDWCQQVKKLSLLGGALPMFELVEQ*

CFP-LC3B-YFP: HisTag—CFP—LC3B—YFP

MHHHHHHMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLV
TTLTWGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDF
KEDGNILGHKLEYNYISHNVYITADKQKNGIKANFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNH
YLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYKGSMPSEKTFKQRRTFEQRVEDVRLIREQHP
TKIPVIIERYKGEKQLPVLDKTKFLVPDHVNMSELIKIIRRRLQLNANQAFFLLVNGHSMVSVSTPISE
VYESEKDEDGFLYMVYASQETFGMKLSVKLMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDAT
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TRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSV
QLADHYQQNTPIGDGPVLLPDNHYLSYQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK*