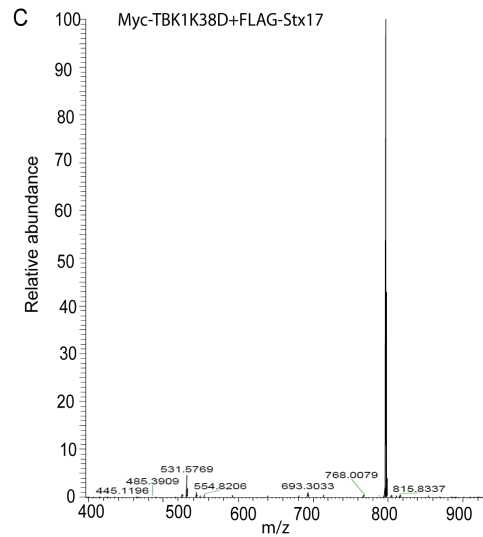
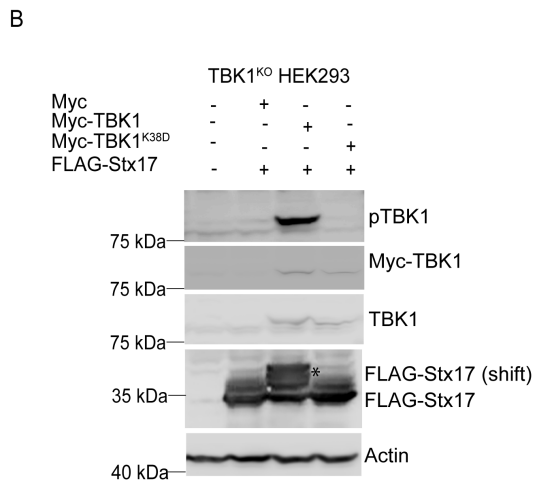


**A** **TBK1\_HUMAN (100%), 83,644.7 Da**  
**Serine/threonine-protein kinase TBK1 OS=Homo sapiens GN=TBK1 PE=1 SV=1**  
**6 exclusive unique peptides, 7 exclusive unique spectra, 7 total spectra, 87/729 amino acids (12% coverage)**

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MQSTSNHLWL  LSDILGQGAT  ANVFRGRGRHKK  TGD LFAIKVF  N N I S F L R P V D
VQ M R E F E V L K  K L N H K N I V K L  F A I E E E T T T R  H K V L I M E F C P  C G S L Y T V L E E
P S N A Y G L P E S  E F L I V L R D V V  G G M N H L R E N G  I V H R D I K P G N  I M R V I G E D G Q
S V Y K L T D F G A  A R E L E D D E Q F  V S L Y G T E E Y L  H P D M Y E R A V L  R K D H Q K K Y G A
T V D L W S I G V T  F Y H A A T G S L P  F R P F E G P R R N  K E V M Y K I I T G  K P S G A I S G V Q
K A E N G P I D W S  G D M P V S C S L S  R G L Q V L L T P V  L A N I L E A D Q E  K C W G F D Q F F A
E T S D I L H R M V  I H V F S L Q Q M T  A H K I Y I H S Y N  T A T I F H E L V Y  K O T K I I S S N Q
E L I Y E G R R L V  L E P G R L A Q H F  P K T T E E N P I F  V V S R E P L N T I  G L I Y E K I S L P
K V H P R Y D L D G  D A S M A K A I T G  V V C Y A C R I A S  T L L L Y Q E L M R  K G I R W L I E L I
K D D Y N E T V H K  K T E V V I T L D F  C I R N I E K T V K  V Y E K L M K I N L  E A A E L G E I S D
I H T K L L R L S S  S O G T I E T S L Q  D I D S R L S P G G  S L A D A W A H Q E  S L A D A W A H Q E
K L Q V L L C N C M T  E I Y Y Q F K K D K  A E R R L A Y N E E  Q I H K F D K Q K L  Y Y H A T K A M T H
F T D E C V K K Y E  A F L N K S E E W I  R K M L H L R K Q L  L S L T N Q C F D I  E E E V S K Y Q E Y
T N E L Q E T L P Q  K M F T A S S G I K  H T M T P I Y P S S  N T L V E M T L G M  K K L K E E M E G V
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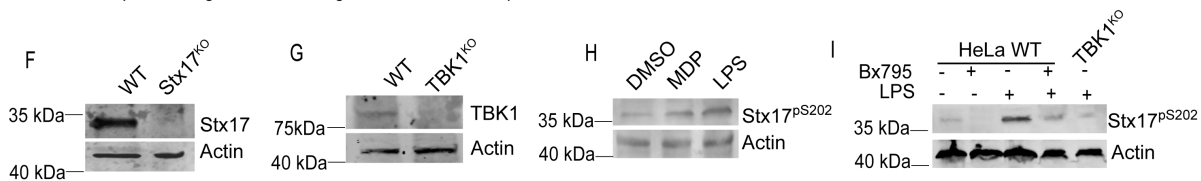
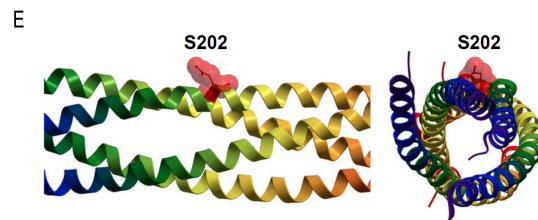
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**D**

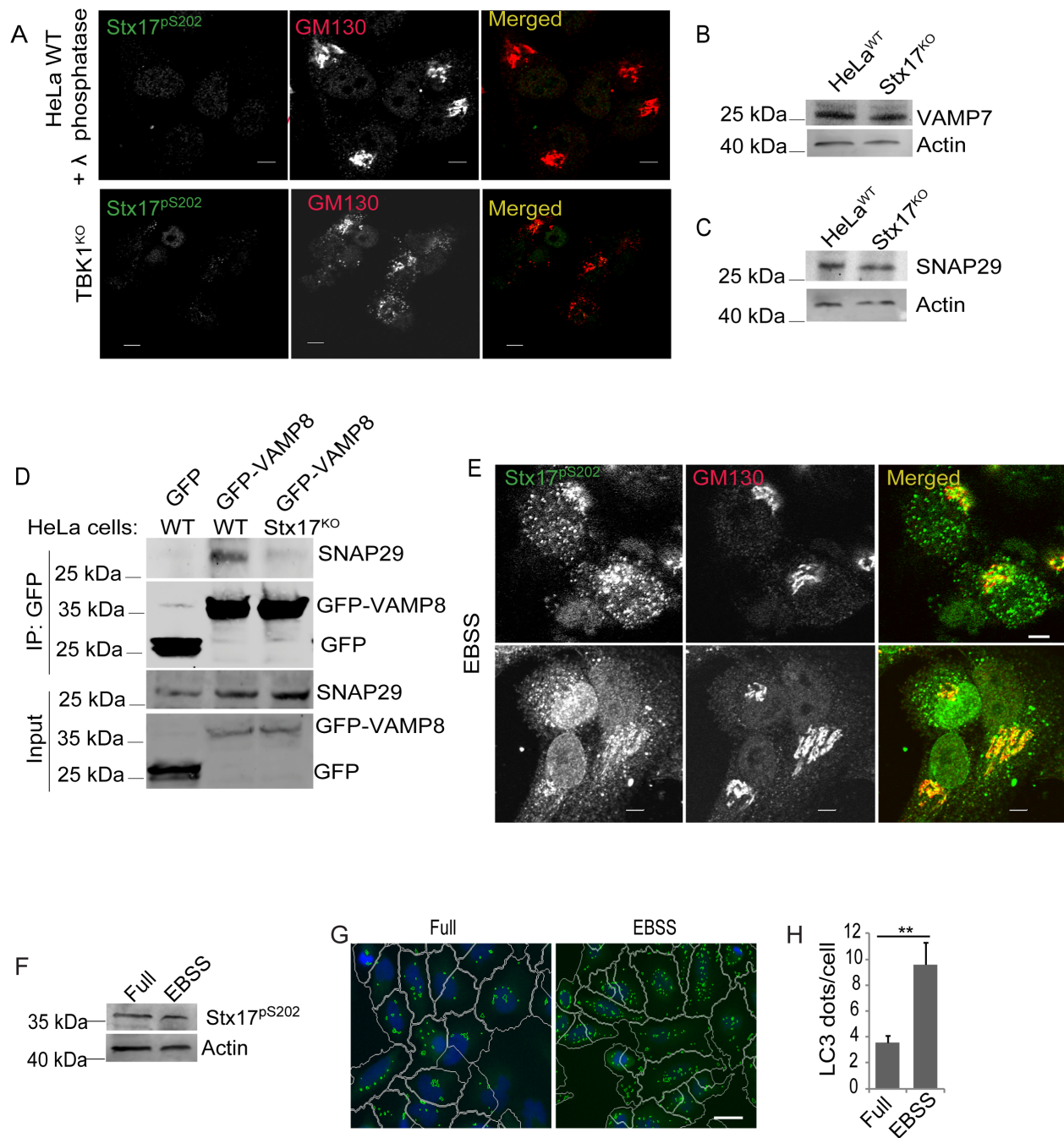
	181	218
H.sapiens	ELS QLVTDFSL LVNSQQEKIDSIADHVNSAAVNVEEGT	
P.trogodytes	ELS QLVTDFSL LVNSQQEKIDSIADHVNSAAVNVEEGT	
M.mulatta	ELS QLVTDFSL LVNSQQEKIDSIADHVNSAAVNVEEGT	
M.musculus	ELSHLVTDM SLLVSSQQEKIDSIADHVNSAAVNVEEGT	
R.norvegicus	ELSHLVTDM SLLVNSQQEKIDSIADHVNSAAVNVEEGT	
D.rerio	QLNGLVNEFSTIVYAQQEKIDSI EANVSIAAANVEEGT	

Sequence alignment showing S202 in different species



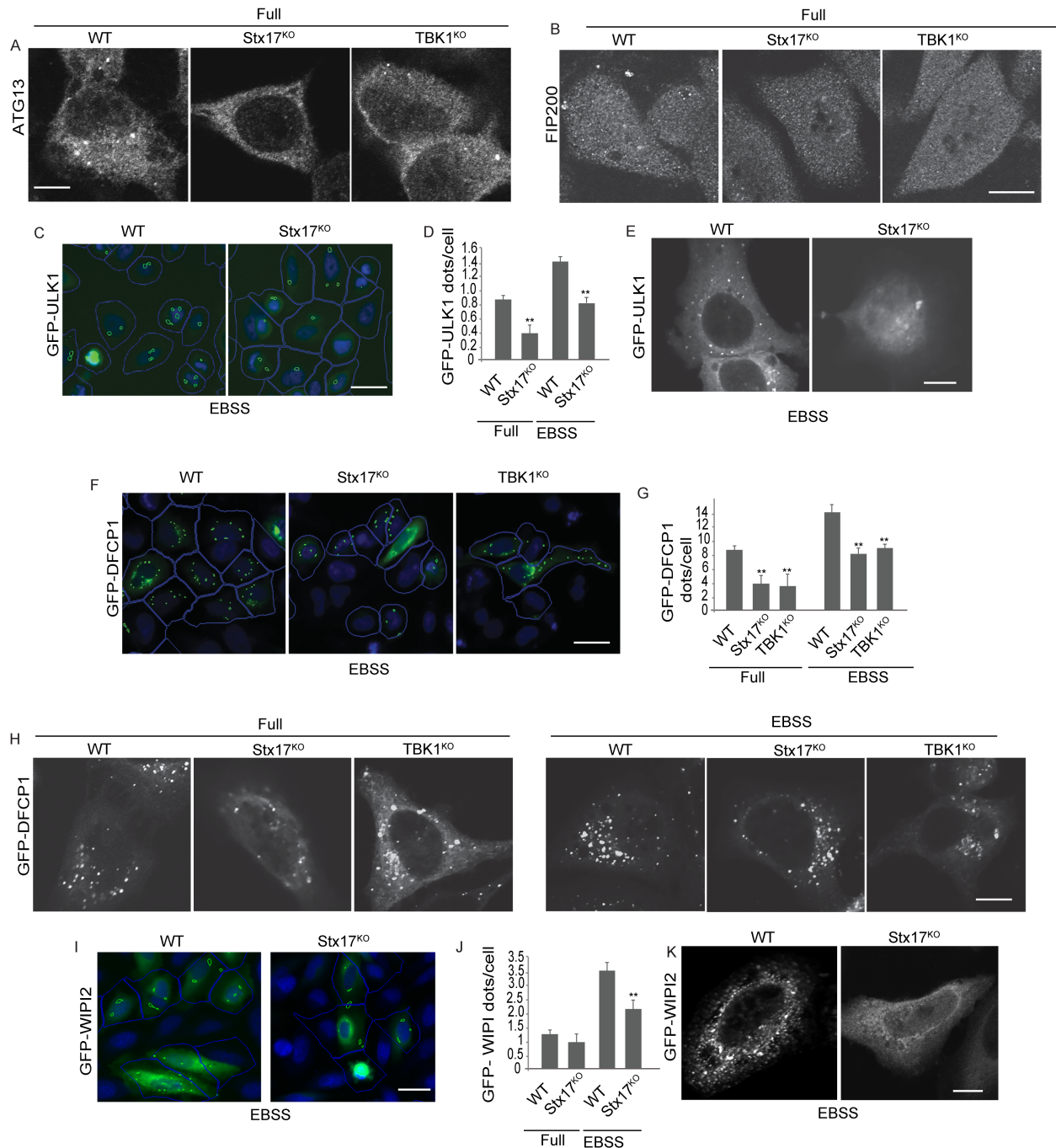
**Figure S1, related to Figure 1. TBK1 phosphorylates Stx17. (A)** A screenshot captured from the scaffold software indicating number of TBK1 peptides interacting with GFP-Stx17 in proteomics study (related to Figure 1A). **(B)** Western blot from TBK1<sup>KO</sup> 293T cells expressing Myc, Myc-TBK1<sup>WT</sup> or Myc-TBK1<sup>K38D</sup> with FLAG-Stx17. “\*” represents phosphorylation-induced shift. **(C)** MS analysis from TBK1<sup>KO</sup> 293T cells to analyze the effect of Myc-TBK1<sup>K38D</sup> on FLAG-Stx17 phosphorylation (related to Figure 1C). **(D)** Sequence alignment showing conserved S202 residue in Stx17 from human to

fish. **(E)** Crystal structure from database showing location of S202 in Stx17. **(F)** Western blot confirming Stx17 knock out in Stx17<sup>KO</sup> HeLa cells. **(G)** Western blot showing TBK1 knock out in TBK1<sup>KO</sup> HeLa cells. **(H)** Western blot to analyze the effect of MDP or LPS on expression of Stx17<sup>pS202</sup> in HeLa cells. **(I)** Western blot to analyze the effect of TBK1 agonist LPS and inhibitor BX795 on expression of Stx17<sup>pS202</sup> in HeLa<sup>WT</sup> or TBK1<sup>KO</sup> cells.



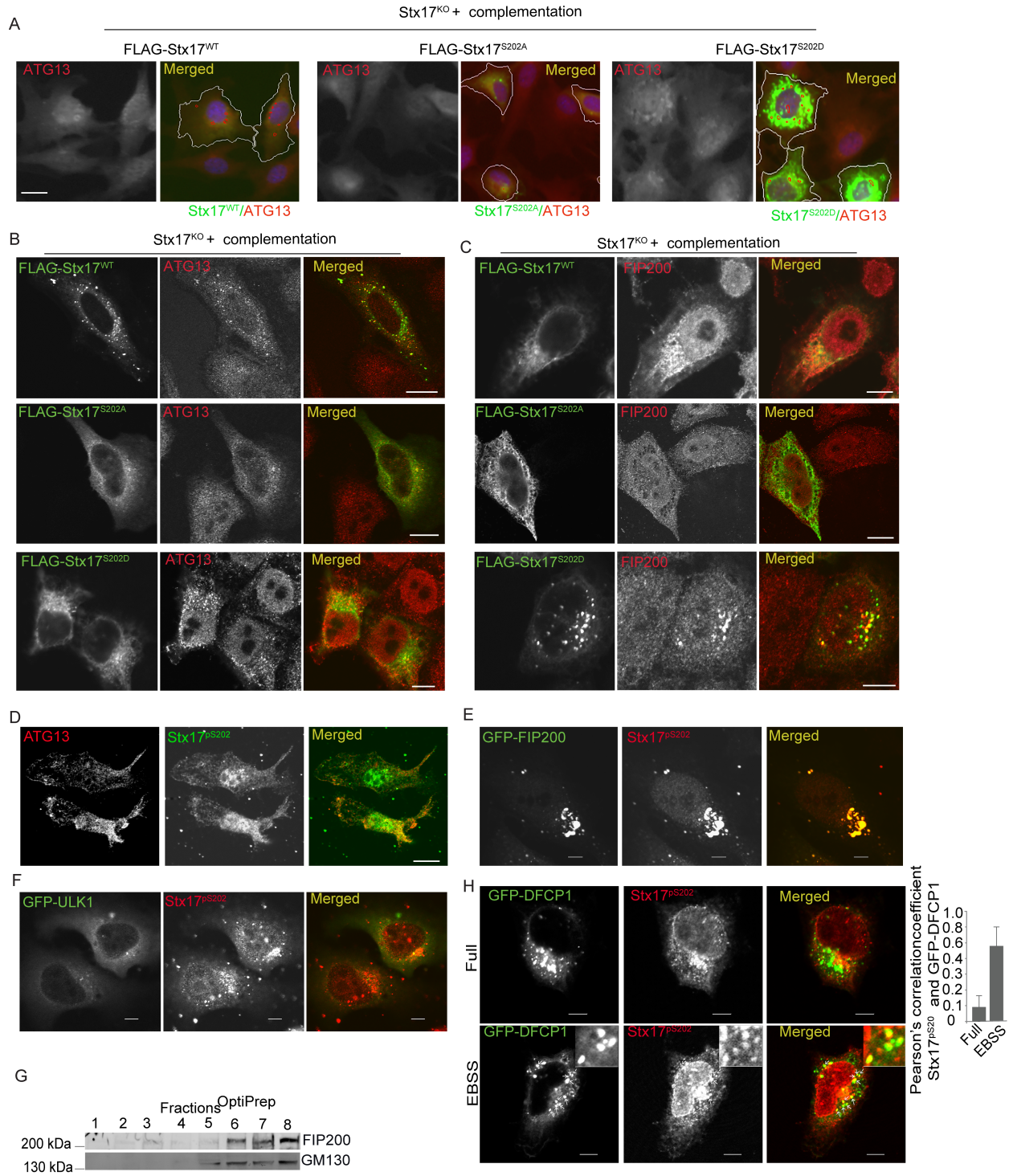
**Figure S2, related to Figure 2. Stx17<sup>pS202</sup> is localized in Golgi. (A) Wild type HeLa cells were treated with λ phosphatase (upper row), while TBK1<sup>KO</sup> HeLa cells (lower row) were left untreated and and stained with Stx17<sup>pS202</sup> and GM130. Colocalization between Stx17<sup>pS202</sup> and GM130 was analyzed by confocal microscopy. Scale bar 5 μm. (B, C) Western blot to analyze the effect of Stx17<sup>KO</sup> on stability of VAMP7 or SNAP29. (D) Co-IP analysis of interaction between GFP-VAMP8 and SNAP29 in HeLa WT or Stx17 KO cells. (E) Confocal microscopy to analyze the distribution of Stx17<sup>pS202</sup> from Golgi to peripheral dots in response to autophagy induction by EBSS. GM130 is used to stain Golgi. Scale bar 5 μm. (F-H) Western blot showing effect of starvation (2h EBSS)**

induced autophagy (as shown by induction of LC3 dots in C,D) on levels of Stx17<sup>pS202</sup>.  
White masks, algorithm-defined cell boundaries (primary objects); green masks,  
computer-identified LC3 dots. \*\*,  $p < 0.01$ , (n=3) t-test.



**Figure S3, related to Figure 3. Stx17 and TBK1 are required for formation of pre-autophagosomal structures.** (A, B) Confocal microscopy analysis of effects of Stx17<sup>KO</sup> and TBK1<sup>KO</sup> on formation of ATG13 and FIP200 dots. Scale bar 5  $\mu$ m. (C, D) HC analysis of effect of Stx17<sup>KO</sup> on formation of ULK1-GFP dots in full media or cells induced for autophagy by incubating in EBSS for 1h. \*\*,  $p < 0.01$ , (n=3) ANOVA. Blue masks, algorithm-defined ULK-GFP positive cells (primary objects); green masks, computer-identified ULK1-GFP dots. (E) Confocal microscopy to analyze the effect of Stx17<sup>KO</sup> on formation of ULK1-GFP dots. Scale bar 5  $\mu$ m. (F, G) High content microscopy and quantifications showing effect of Stx17 and TBK1 knock outs on

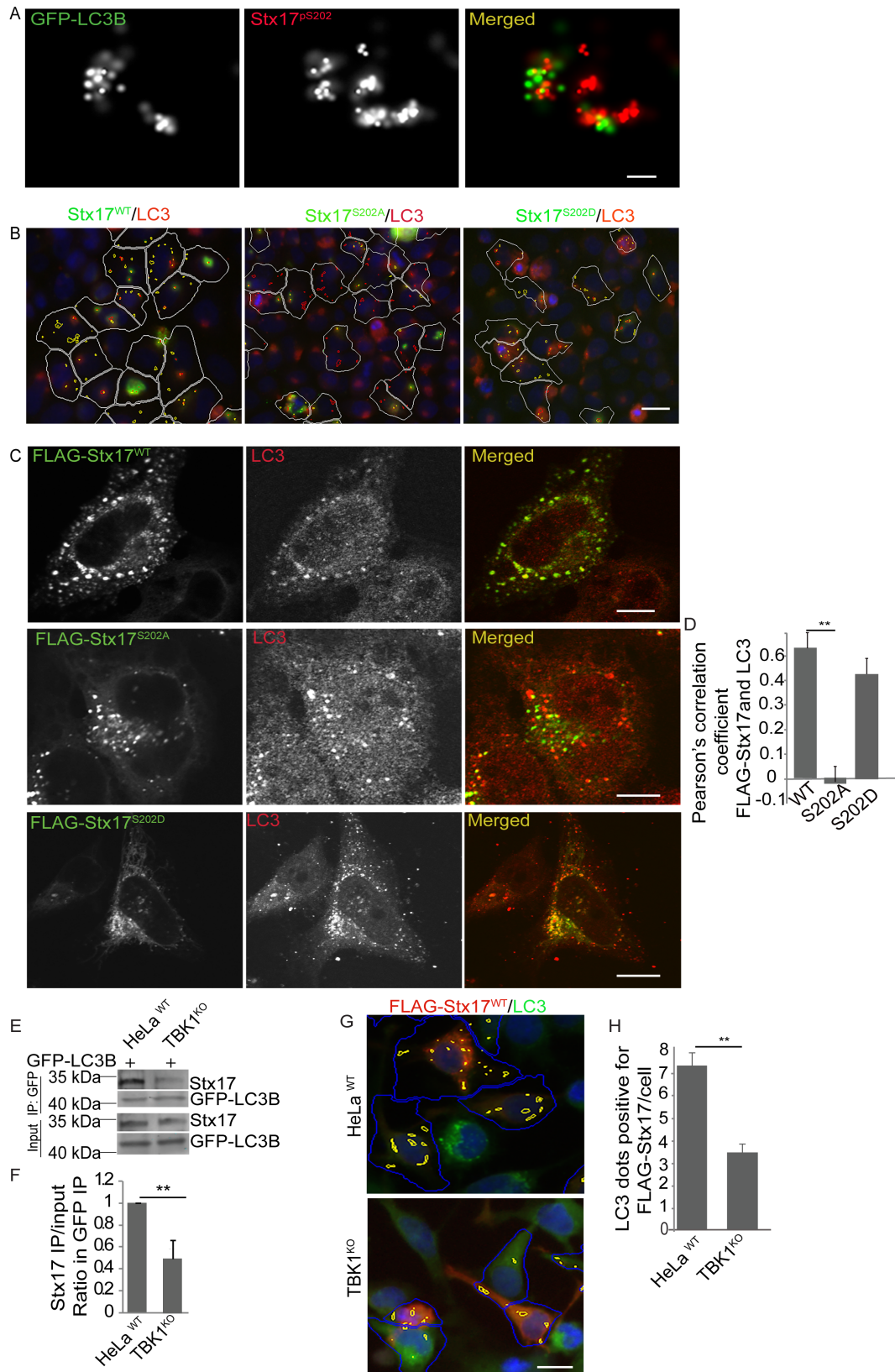
formation of GFP-DFCP1 dots in cells incubated in full media or induced for autophagy by incubating in EBSS for 1h. \*\*,  $p < 0.01$ , (n=3) ANOVA. Blue masks, algorithm-defined GFP-DFCP1 positive cells (primary objects); green masks, computer-identified GFP-DFCP1 dots. **(H)** Confocal microscopy to analyze the effect of Stx17 and TBK1 knock outs on formation of GFP-DFCP1 dots in cells incubated with full media or induced for autophagy by incubating with EBSS for 1h. **(I, J)** High content analysis showing effect of Stx17<sup>KO</sup> on formation of GFP-WIPI2 dots in full media or cells induced for autophagy by incubating in EBSS for 1h. Blue masks, algorithm-defined GFP-WIPI2 positive cells (primary objects); green masks, computer-identified GFP-WIPI2 dots. \*\*,  $p < 0.01$ , (n=3) ANOVA. **(K)** Confocal microscopy to analyze the effect of Stx17 and TBK1 knock outs on formation of GFP-WIPI2 dots.



**Figure S4, related to Figure 4 and Figure 5. Stx17<sup>pS202</sup> colocalizes with mPAS. (A)** HC images illustrating the effect of complementation Stx17<sup>KO</sup> cells with FLAG Stx17<sup>WT</sup>, Stx17<sup>S202A</sup> and Stx17<sup>S202D</sup>. Masks: white; FLAG positive cells selected by the machine, red; ATG13 dots in FLAG transfected cells (merged images). Black and white images show unmasked epifluorescence images. White masks, algorithm-defined FLAG positive cells (primary objects); red masks, computer-identified ATG13 dots in FLAG

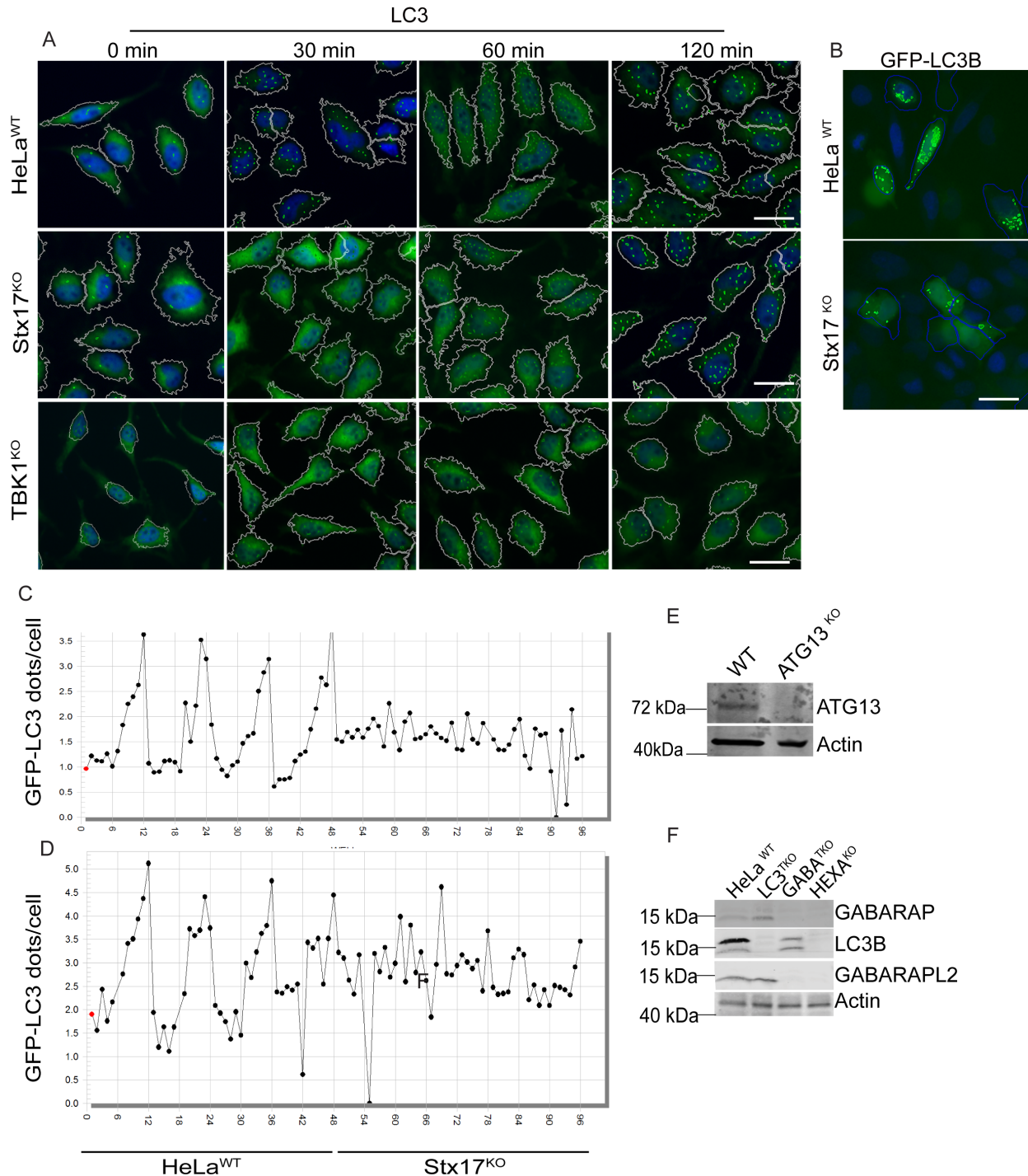
positive cells. **(B, C)** Confocal microscopy to analyze the effect of complementation of Stx17KO cells with FLAG Stx17<sup>WT</sup>, Stx17<sup>S202A</sup> and Stx17<sup>S202D</sup> on formation of ATG13 (B) or FIP200 (C) dots. Scale bar 5  $\mu\text{m}$ . **(D)** Confocal microscopy analysis of colocalization between ATG13 and Stx17<sup>pS202</sup> in mouse BMMs. Scale bar 5  $\mu\text{m}$ . **(E)** Confocal microscopy to analyze the colocalization between GFP-ULK1 in and Stx17<sup>pS202</sup> in HeLa cells grown in full media. Scale bar 5  $\mu\text{m}$ . **(F)** 293T cells were subjected to differential centrifugation and 25 k samples were layered on Optiprep gradients as described in materials and methods. Optiprep fractions were subjected to immunoblotting to analyze co-fractionation of FIP200 with Golgi marker GM130. **(G)** Confocal microscopy analysis of colocalization between Stx17<sup>pS202</sup> and ULK-GFP in HeLa cell grown in full media. Scale bar 5  $\mu\text{m}$ . **(H)** Confocal microscopy analysis of colocalization between GFP-DFCP1 and Stx17<sup>pS202</sup> in HeLa cells. Cells were left in full media (upper row) or incubated with EBSS for 1h (lower row). Arrows indicate Stx17<sup>pS202</sup> and GFP-DFCP1 dots overlapping with each other. Scale bar 5  $\mu\text{m}$ . **(I)** Pearson's correlation coefficient (>20 cells) of colocalization between GFP-DFCP1 and Stx17<sup>pS202</sup>.





**Figure S5, related to Figure 6. WT and phosphomimetic but not non-phosphorylatable Stx17 colocalizes with LC3. (A)** Super-resolution microscopy to

analyze the colocalization between GFP-LC3B and Stx17<sup>pS202</sup> in HeLa cells incubated with EBSS for 2h. Scale bar 500nm. **(B)** HC microscopy to analyze colocalization between FLAG-tagged Stx17<sup>WT</sup>, Stx17<sup>S202A</sup> or Stx17<sup>S202D</sup> mutants with LC3 in cell induced for autophagy with starvation. Scale bar 10  $\mu$ m. White masks, algorithm-defined FLAG-Stx17 positive cells (primary objects); yellow masks, computer-identified overlap between FLAG-Stx17 and LC3; red, LC3<sup>+</sup> FLAG<sup>-</sup> dots. **(C)** Confocal Microscopy to analyze colocalization between FLAG-tagged Stx17<sup>WT</sup>, Stx17<sup>S202A</sup> or Stx17<sup>S202D</sup> mutants with LC3 in cell induced for autophagy with starvation. Scale bar 5  $\mu$ m. **(D)** Graph showing Pearson's correlation coefficient of colocalization between FLAG-tagged Stx17 variants and LC3. **(E, F)** Co-IP analysis and quantifications of interactions between Stx17 and GFP-LC3B in HeLa<sup>WT</sup> or TBK1<sup>KO</sup> cells. p<0.05; \*\*, p < 0.01, (n=3) t-test. **(G,H)** HC analysis and quantifications of colocalization between FLAG-Stx17 and LC3 in HeLa<sup>WT</sup> or TBK1<sup>KO</sup> cells induced for autophagy by incubating with EBSS for 1h. Blue masks, algorithm-defined FLAG-Stx17 positive cells (primary objects); yellow masks, computer-identified overlap between FLAG-Stx17 and LC3. p<0.05; \*\*, p < 0.01, (n=3) t-test.



**Figure S6, related to Figure 7. Stx17 regulates autophagy initiation (A)** HC microscopy to analyze the effect of Stx17 and TBK1 knock out on LC3 puncta formation at indicated time points of autophagy induction by starvation. White masks, algorithm-defined cell boundaries (primary objects); green masks, computer-identified LC3 dots. **(B)** High content images showing effect of Stx17 knock out on GFP-LC3 puncta formation after 1h autophagy induction with EBSS. White masks, algorithm-defined GFP-LC3 positive cells (primary objects); green masks, computer-identified GFP-LC3B dots. **(C, D)** Screenshots of layout of the plates used in Figures 7J and S7B, showing

effect of Stx17 knock outs (right half of the plates) on formation of GFP-LC3B puncta. **(E)** Western blot showing ATG13 knock out in HeLa cells. **(F)** Western blot confirming LC3B, GABRAP and GABARAPL2 knock outs in mATG8s knock out cells.