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Supplemental Material to:

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Autophagy facilitates organelle clearance during differentiation of human erythroblasts: Evidence for a role for ATG4 paralogs during autophagosome maturation

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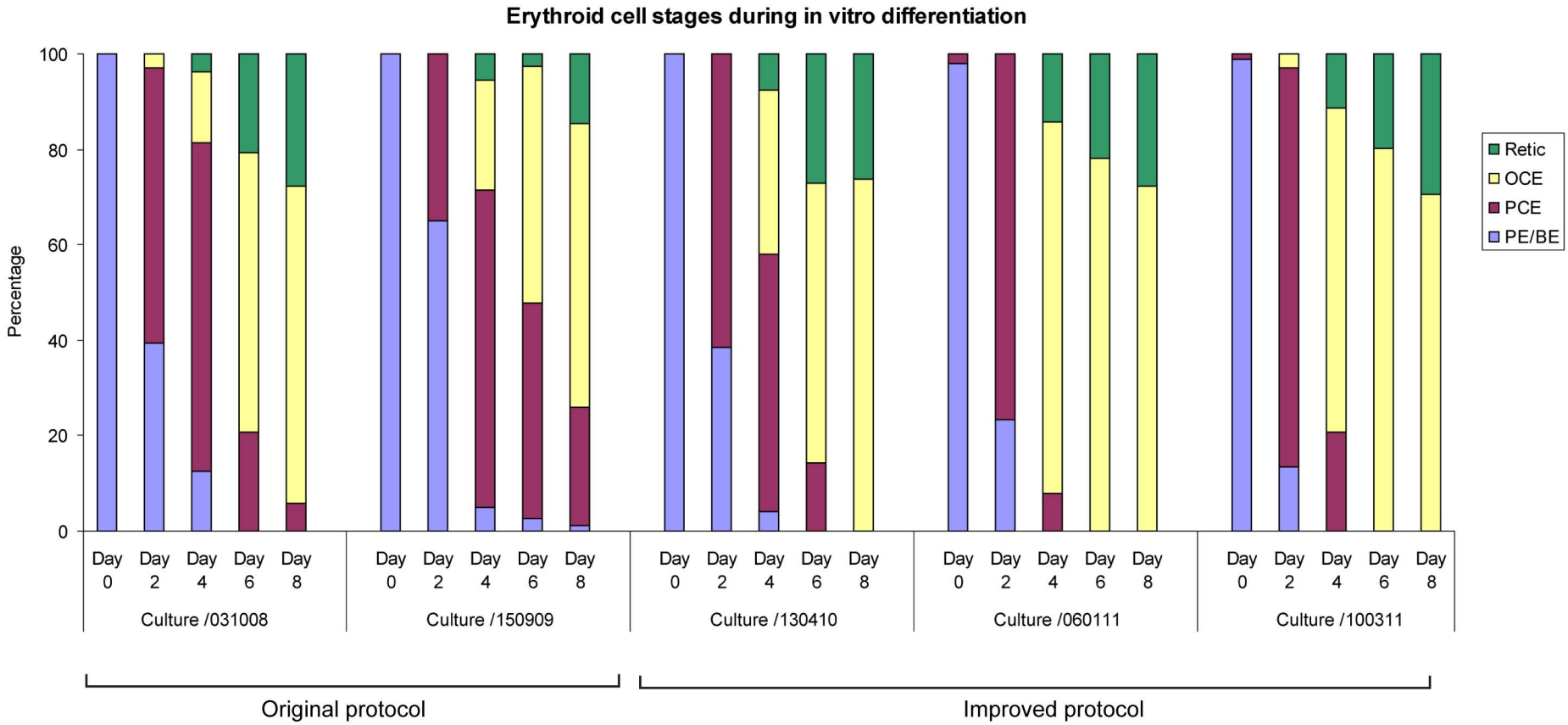
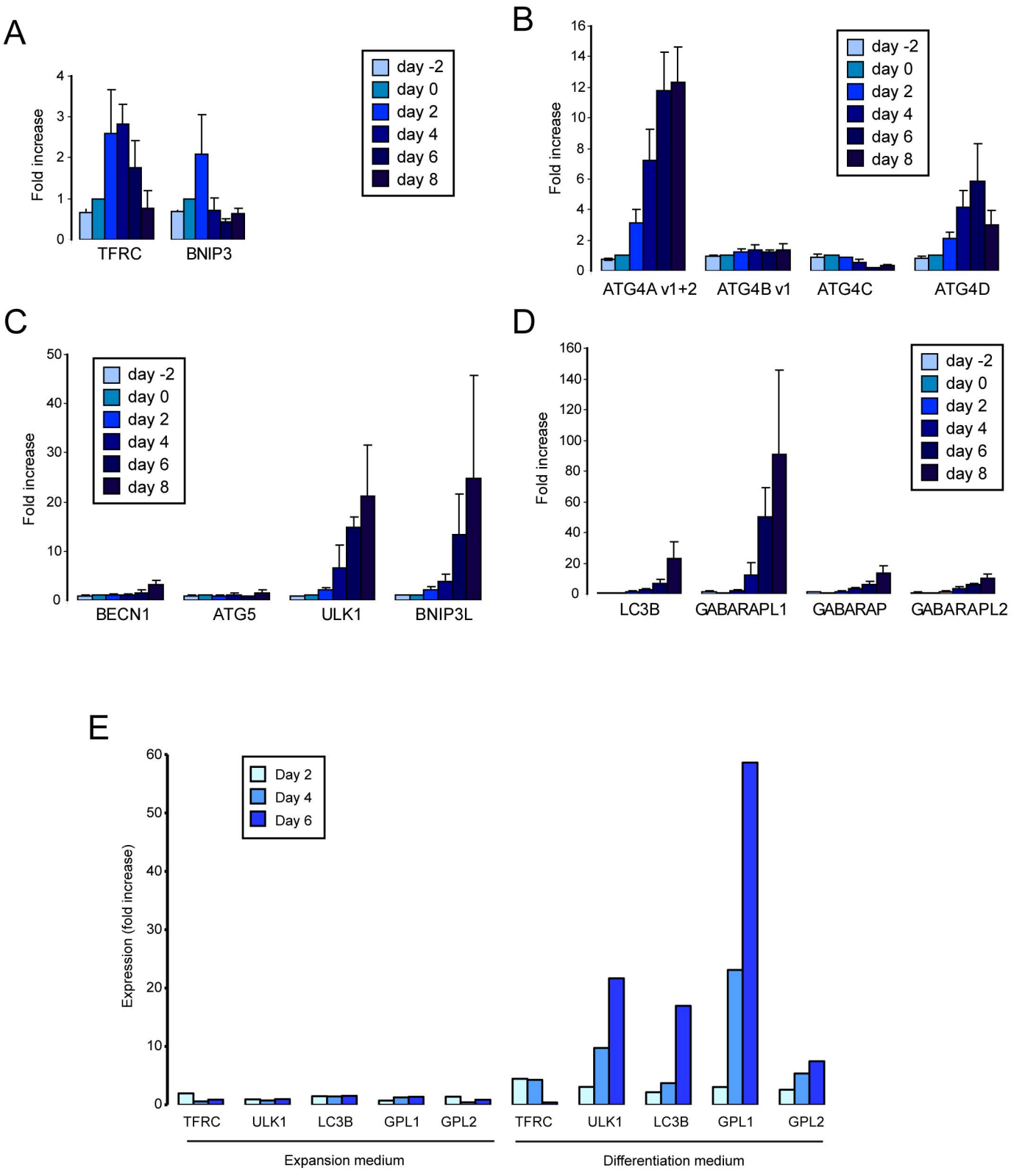
Supplemental Table 1: Primer design and conditions for RT-PCR

	Primer name	Target	Primer sequence	%GC	PCR product	Genbank ref	Primers (nM)	Annealing temp
General Autophagy Genes	RT-PABPC1 For	Poly(A) binding protein, cytoplasmic 1	agctgttcccaaccctgtaatc	50	101 bp	NM_002568	300 nM	60°C
	RT-PABPC1 Rev	Poly(A) binding protein, cytoplasmic 1	ggatagtatgcagcacggttctg	52				
	RT-Beclin1-For	BECN1	aggttgagaaaggcgagaca	50	196 bp	NM_003766	300 nM	60°C
	RT-Beclin1-Rev	BECN1	aattgtgaggacacccaagc	50				
	RT-Atg5-For	Atg5	aagcaactctggatgggattg	50	236 bp	NM_004849	150 nM	60°C
	Rt-Atg5-Rev	Atg5	caggatcaatagcagaaggac	48				
	RT-Bnip3-For	BNIP3	gctcctgggtagaactgcac	60	190 bp	NM_004052	200 nM	58°C
	RT-Bnip3-Rev	BNIP3	gccctgttggtatcttgtgg	55				
	RT-Ulk 1-For	ULK1	ggaagatgtctctgggtgga	55	223 bp	NM_003565	300 nM	60°C
	RT-Ulk 1-Rev	ULK1	acgacgtgcaagtcagacag	55				
	RT-Nix-For	BNIP3L	ccctgcacaacaacaacaac	50	173 bp	NM_004331.2	150 nM	58°C
	RT-Nix-Rev	BNIP3L	attgtggatggaggatgagg	50				
Human Atg8 Paralogs	RT-LC3B For	MAP1LC3B	accatgtcaacatgagtgagc	47.6	207 bp	NM_022818	300 nM	60°C
	RT-LC3B Rev	MAP1LC3B	ctgacaatttcatcccgaacg	47.6				
	RT-GATE-16/GP-L2 F	GABARAPL2	cacagatgcgtggaatccgc	60	233 bp	NM_007285	300 nM	60°C
	RT-GATE-16/GP-L2 R	GABARAPL2	gttaggctggactgtgggac	60				
	RT-GABARAPL1 For	GABARAPL1	ccccgtgattgtagagaagg	55	171 bp	NM_031412	300 nM	60°C
	RT-GABARAPL1 Rev	GABARAPL1	ggagggatggtgttgttgac	55				
	RT-GABARAP For	GABARAP	gaagcgccgctctgag	68.8	182 bp	NM_007278	150 nM	58°C
	RT-GABARAP Rev2	GABARAP	cagctcggagatgaattcg	52.6				
Human Atg4 Paralogs	RT-4Av1+2-For	Atg4A	ctgcagtccccacagcg	70	217 bp	NM_052936 (v1) NM_178270 (v2)	150 nM	60°C
	RT-4Av1+2-Rev	Atg4A	ccccagtggttgtcacttct	55				
	RT-4Bv1-For	Atg4B	ggtgccagcaagtcaaaaag	50	152 bp	NM_013325	300 nM	60°C
	RT-4Bv1-Rev	Atg4B	gtcgaagaatctttccagtcg	47.6				
	RT-4Bv2-For	Atg4B	gacttcaatgattggtgccagc	50	149 bp	NM_178326	150 nM	60°C
	RT-4Bv2-Rev	Atg4B	atctggacttggcagctctc	55				
	RT-4C-2For	Atg4C	cagctgtggttgctcacattt	50	222 bp	NM_178221 (variants 7&8)	150 nM	60°C
	RT-4C-2Rev	Atg4C	ctaagtagtcggtgttggttc	48				
	RT-4D-4For	Atg4D	ccgcgacactcactgtactt	55	202 bp	NM_032885	300 nM	60°C
	RT-4D-4Rev	Atg4D	actccttcctgtctccagca	55				
Control Genes	RT-Actin B For	ACTB	catcaccattggcaatgagc	47.4	282 bp	NM_001101	300 nM	60°C
	RT-Actin B Rev	ACTB	ccacacggagtacttgcgc	63.2				
	RT-CD71-For	TFRC	aaaatccggtgtaggcacag	50	181 bp	NM_003234.2	500 nM	58°C
	RT-CD71-Rev	TFRC	ctttaaatgcagggacgaaagg	45				

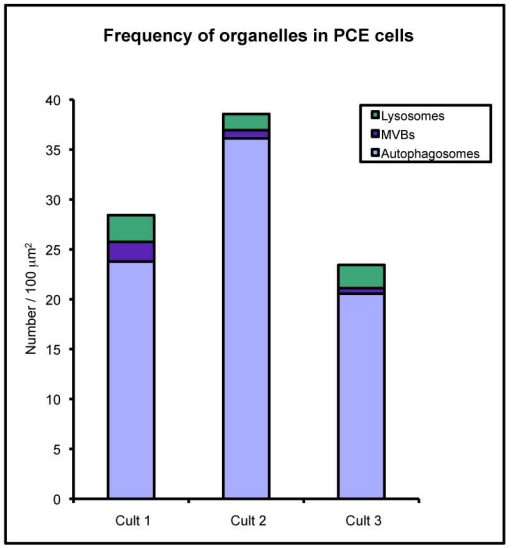
Betin et al. Supplementary Fig. 1

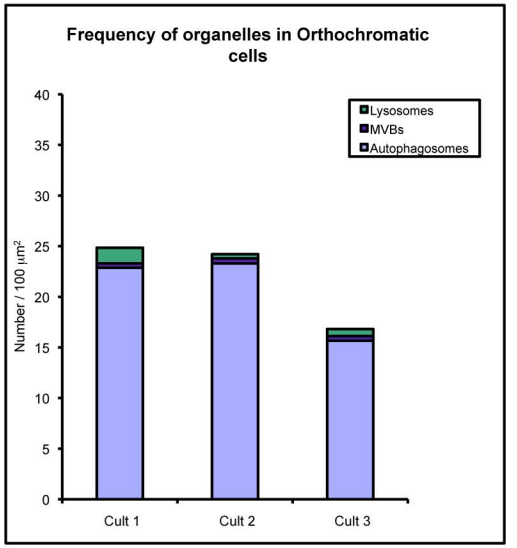


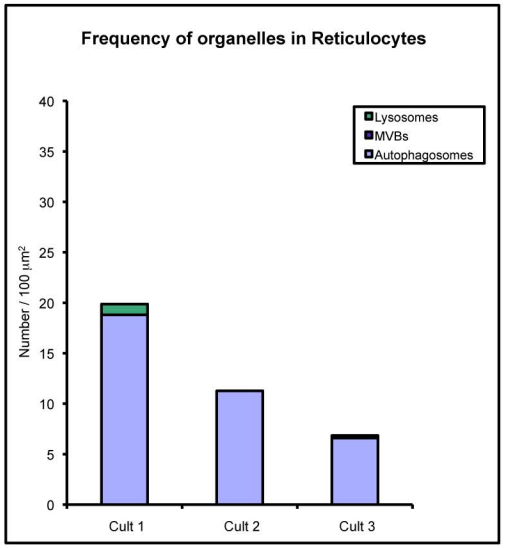
Betin et al. Supplementary Fig. 2

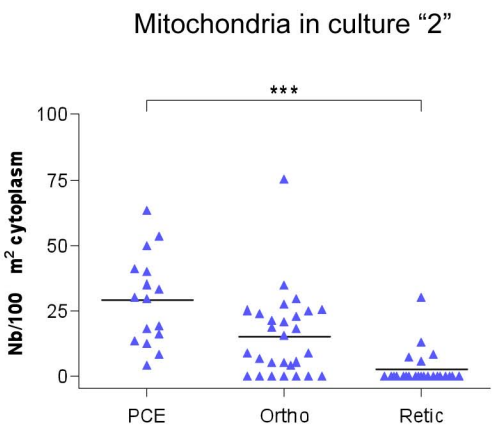


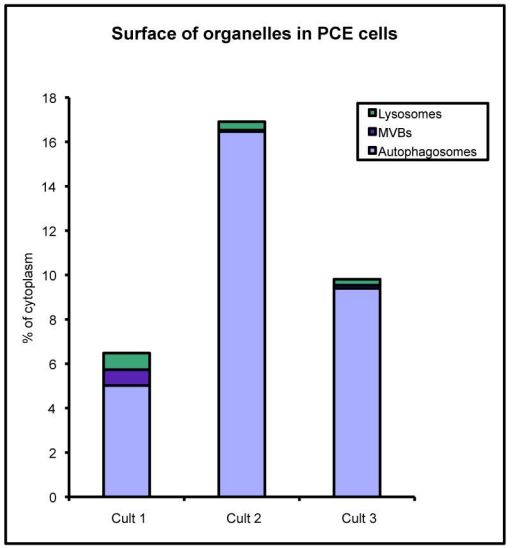
Betin et al. Supplementary Fig. 3

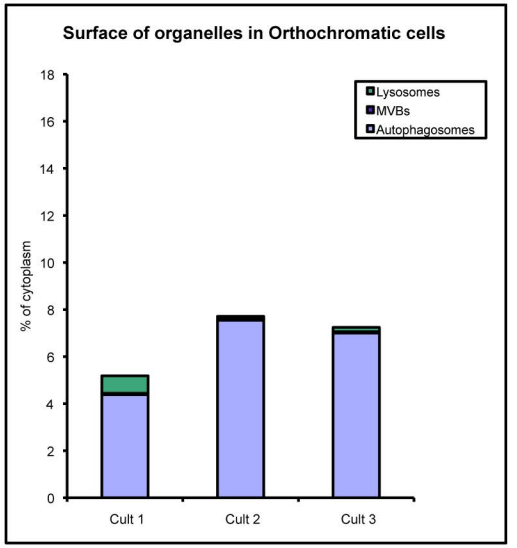


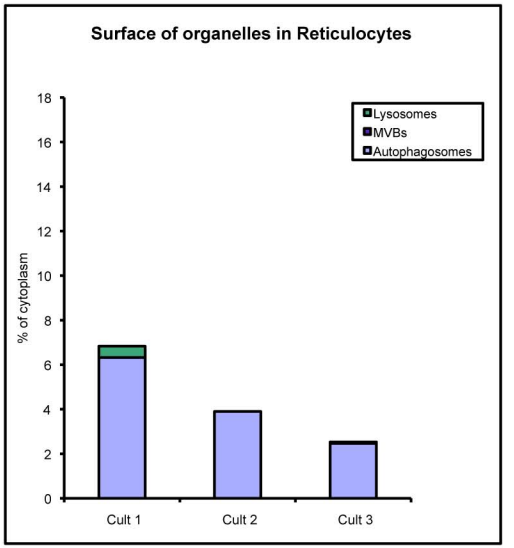


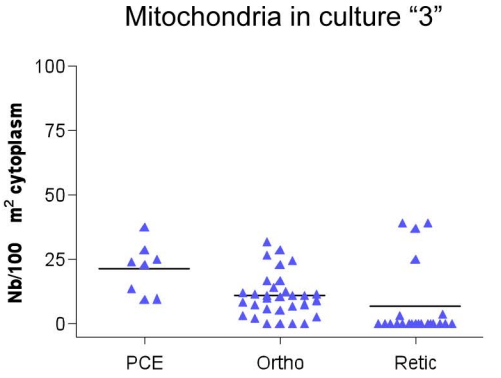




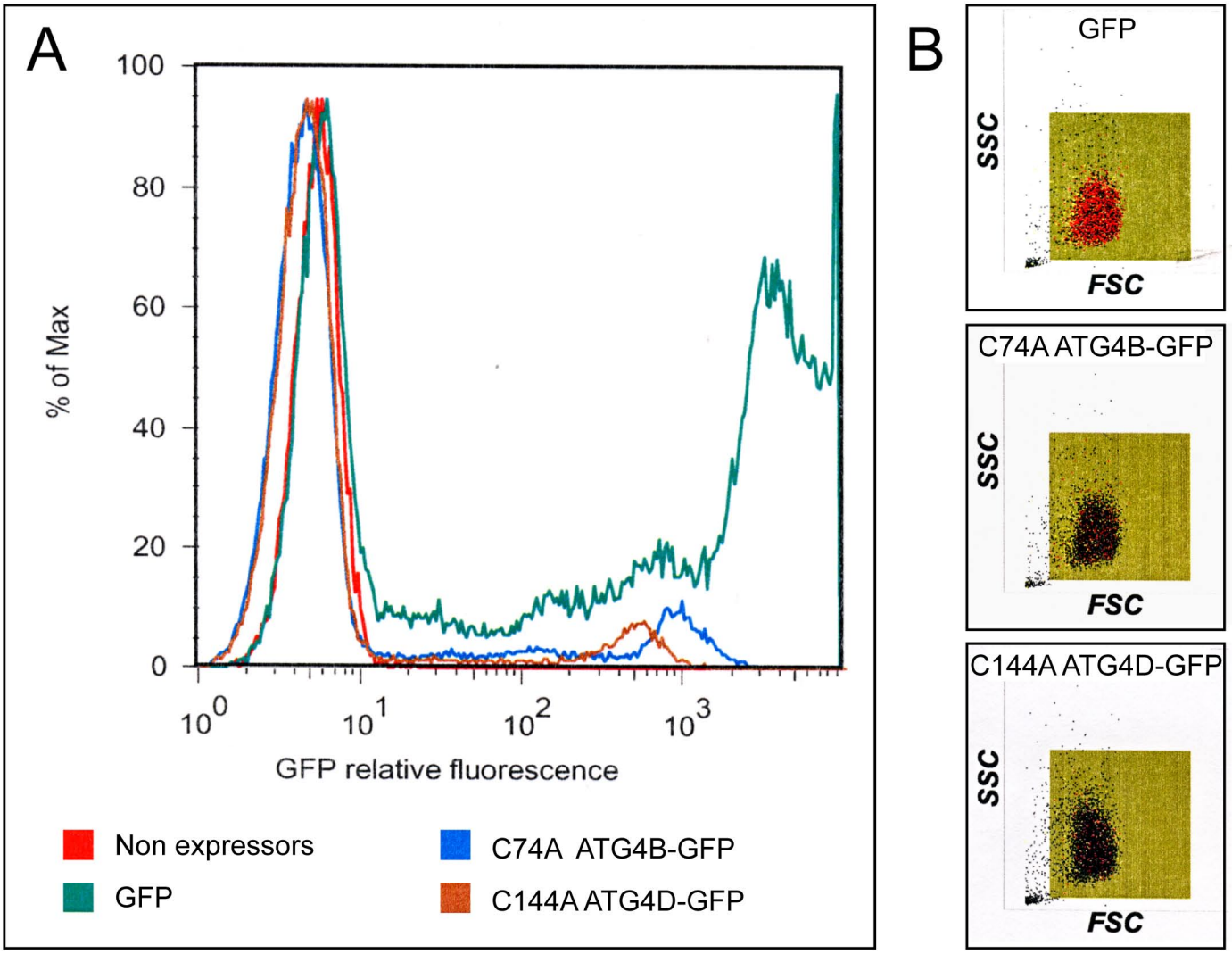








Betin et al. Supplementary Fig. 4



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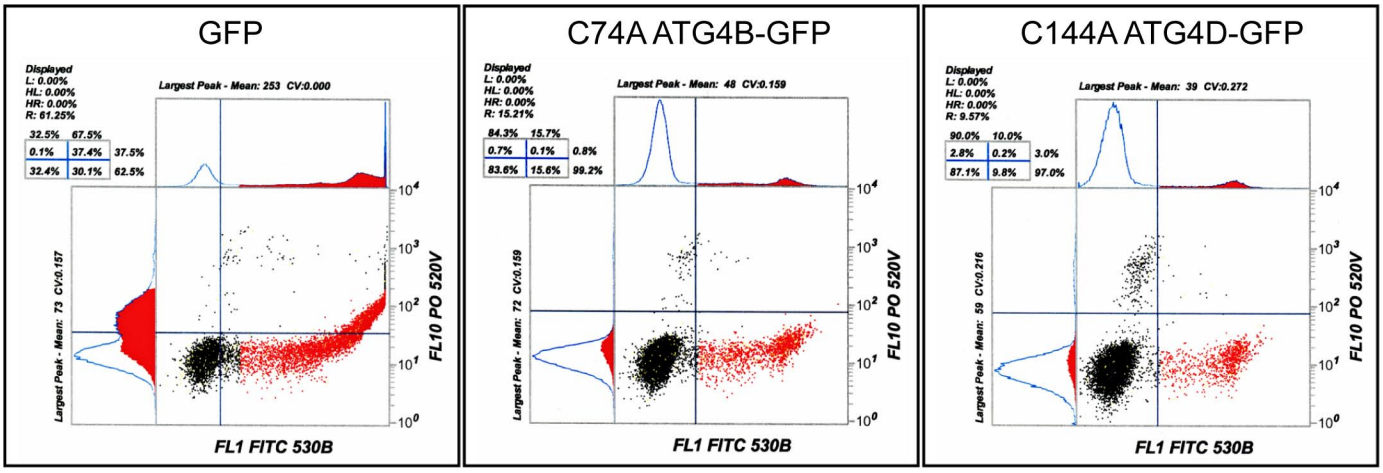
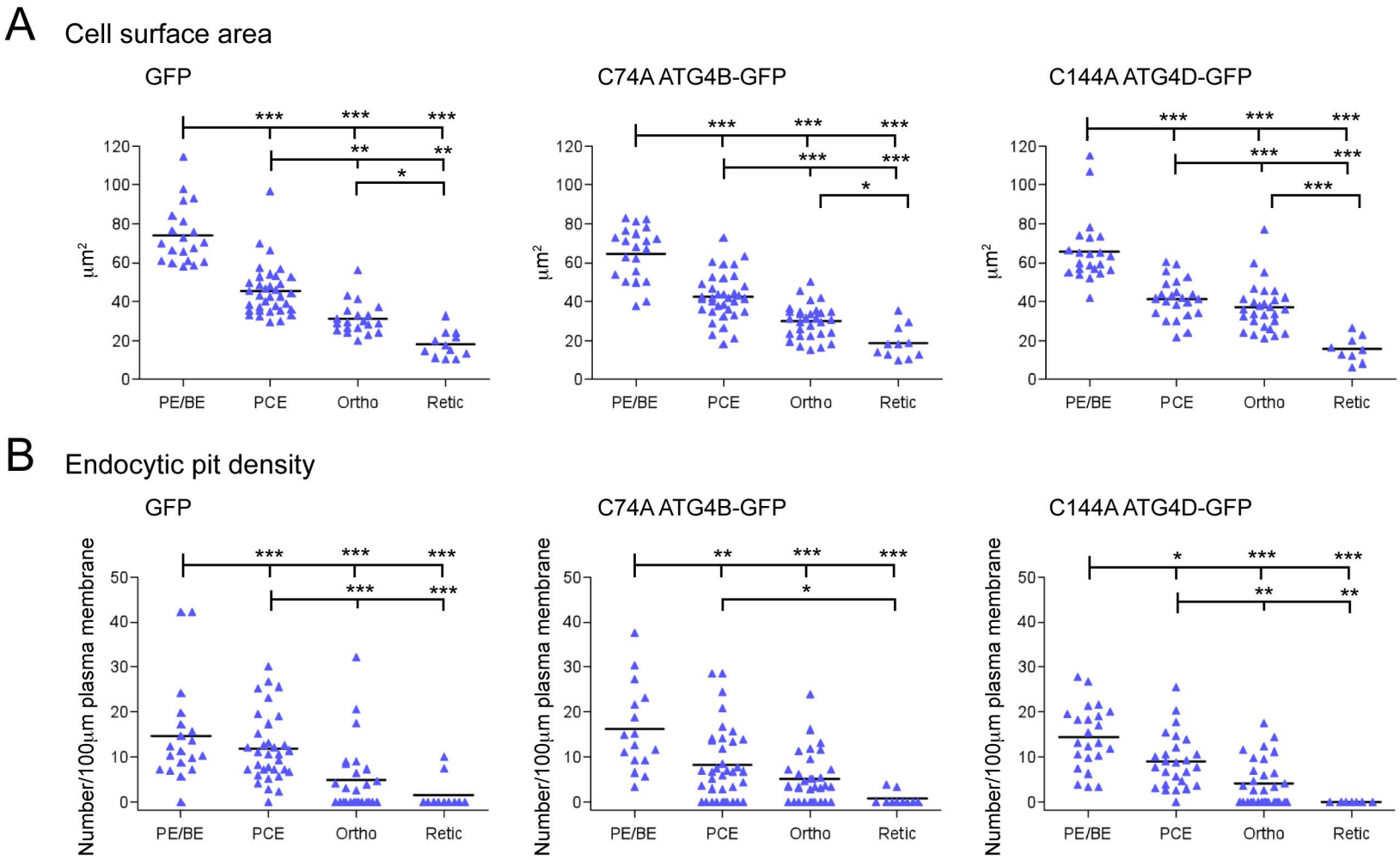
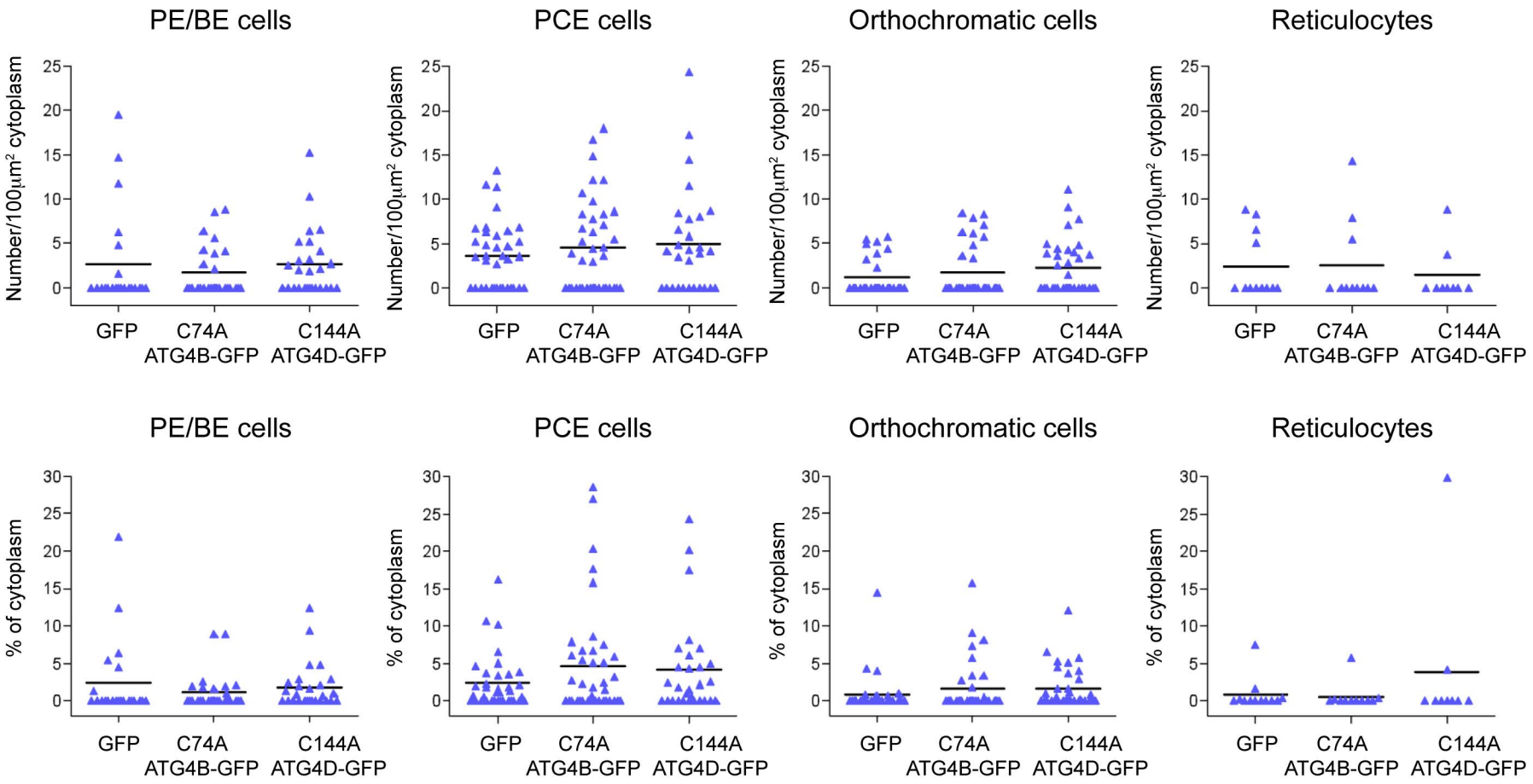


Betin et al. Supplementary Fig. 5



Betin et al. Supplementary Fig. 6

Amphisomes



Betin et al. Supplementary Fig. 7

Lysosomes

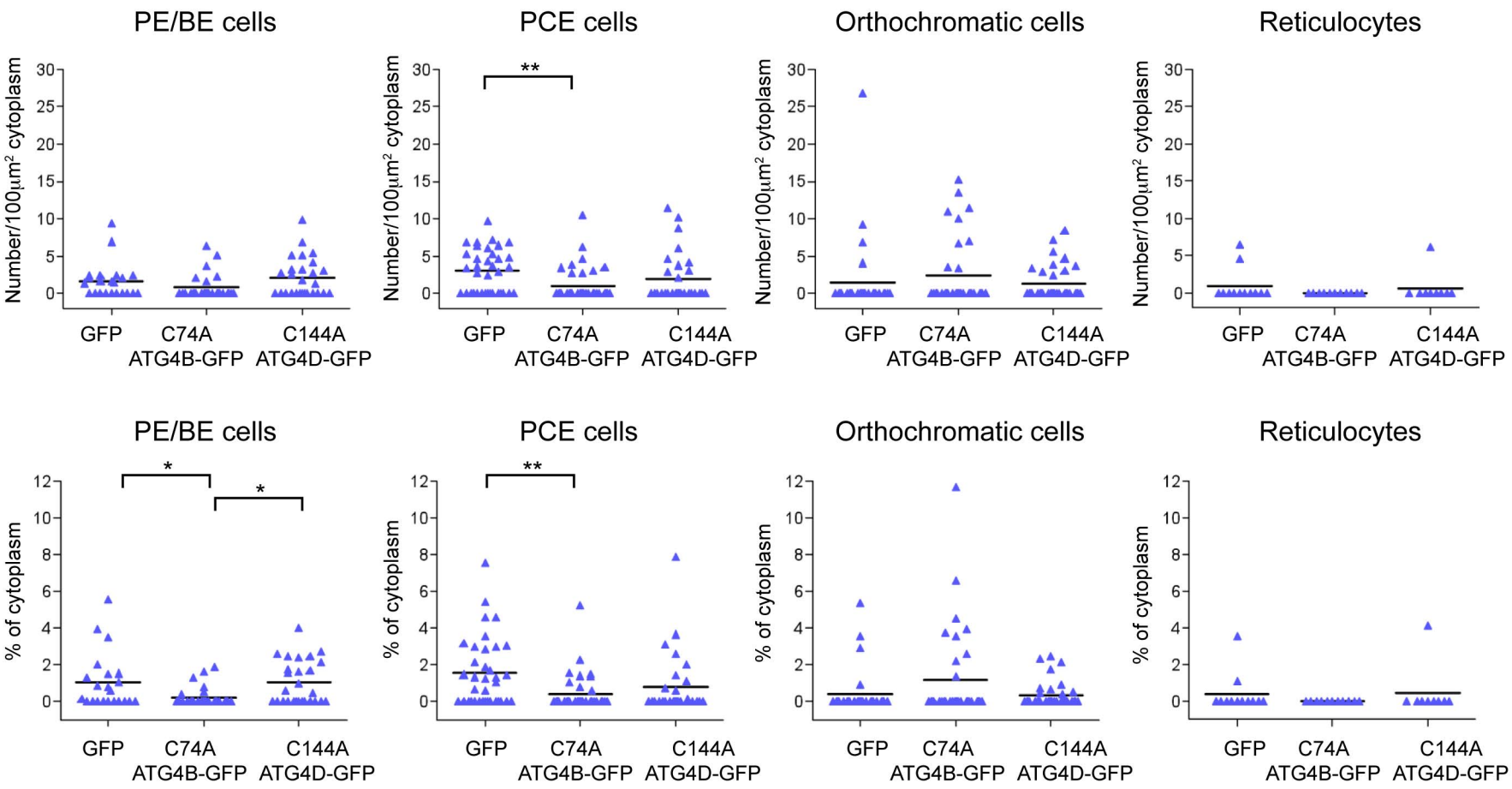


Figure S1. Erythroid terminal differentiation protocols for 5 different cultures from primary human $CD34^+$ cells. Cultures to the left (/031008, /150909) were obtained using an early culture system (following 5 days culture in expansion medium); those to the right (/130410, /060111, /100311) included an extended expansion step (10 days expansion, including 7 days with dexamethasone). This approach produced more uniform cultures with fewer cells stalled at the PCE stage.

Figure S2. Transcriptional regulation of autophagy genes during human erythroid differentiation in vitro. (**A-D**) qRT-PCR analysis of RNA samples collected at 2 day intervals before (-2) and after switch to differentiation media (day 0). Results are expressed as fold-increase compared to day 0. Histograms represent the means ± s.d. of 3 independent experiments (for *TFRC*, *BNIP3L*, *GABARAP* and *ATG4C*) or 4 independent experiments (for all the other genes tested). (**E**) Expression of selected transcripts in cells maintained in expansion medium was compared with expression of cells triggered to terminally differentiate. Values are expressed as a fold-increase in expression using data at day 0 as reference.

Figure S3. Analysis of organelle populations in 3 independent erythroid cultures. Bars show organelle number/100 μm^2 cytoplasmic area (top) and % cytoplasmic occupancy (bottom) of autophagosomes, MVBs and lysosomes in 3 separate cultures. Data were obtained by quantitative morphometric analysis of high pressure frozen samples imaged by electron microscopy. Mitochondrial density for cultures “2” and “3” are also shown. PCE, orthochromatic and reticulocyte stages only are shown.

Figure S4. Pre-sorting flow cytometry analysis of erythroid progenitor cell populations transduced with GFP, GFP-ATG4B^{C74A} or GFP-ATG4D^{C144A} lentiviruses. (**A**) Flow cytometric analysis of transduced erythroid progenitors prior to cell sorting at day 7 of culture. GFP alone (green line), GFP-ATG4B^{C74A} (blue line), GFP-ATG4D^{C144A} (orange line), and non-transduced controls (red line). (**B**) Unaltered cellular volume (forward scatter: FSC) and granulosity (side scatter: SSC) in erythroid

progenitors expressing GFP-tagged proteins (in red) compared to non-expressing cells (in black). (**C**) A population of progenitor cells positive for GFP (FL1-FITC530B) was selected in each culture (gated population in red) and sorted from non-expressing or weakly expressing cells (in black).

Figure S5. Cross sectional surface area and endocytic pit density in human erythroid cells overexpressing GFP, C74A GFP-ATG4B^{C74A} or GFP-ATG4D^{C144A}. Number of cells analysed respectively in PE/BE, PCE, orthochromatic and reticulocyte stages: GFP – 22, 36, 31, 12; GFP-ATG4B^{C74A} – 28, 40, 35, 11; GFP-ATG4D^{C144A} – 27, 29, 35, 9. Black bars represent the means. Tukey's multiple comparison test: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure S6. Numbers and cytoplasmic volume occupancy of amphisomes in human erythroid cells overexpressing GFP, GFP-ATG4B^{C74A} or GFP-ATG4D^{C144A}. Number of cells analysed respectively in PE/BE, PCE, orthochromatic and reticulocyte stages: GFP – 22, 36, 31, 12; GFP-ATG4B^{C74A} – 28, 40, 35, 11; GFP-ATG4D^{C144A} – 27, 29, 35, 9. Black bars represent the means. Tukey's multiple comparison test: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure S7. Numbers and cytoplasmic volume occupancy of lysosomes in human erythroid cells overexpressing GFP, GFP-ATG4B^{C74A} or GFP-ATG4D^{C144A}. Number of cells analysed respectively in PE/BE, PCE, orthochromatic and reticulocyte stages: GFP – 22, 36, 31, 12; GFP-ATG4B^{C74A} – 28, 40, 35, 11; GFP-ATG4D^{C144A} – 27, 29, 35, 9. Black bars represent the means. Tukey's multiple comparison test: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.