Selective integrin endocytosis is driven by interactions between the integrin α-chain and AP2

- Supplementary Figures 1-5 and associated figure legends
- Supplementary Data Set 1: uncropped blots
- Legends for Supplementary Movies 1-5

a

+ Yxxϕ Motif	- Yxxϕ Motif
ITA2	ITA1
ITAD	ITA10
ITAM	ITA11
ITAX	ITAL
ITA7	ITA2B
ITA6	ITA5
ITA9	ITA8
ITA3	ITAV
ITA4	
ITAE	

Homo Sapiens

+ Yxxϕ Motif	- Yxxϕ Motif
ITA2	ITA1
ITAD	ITA10
ITAM	ITA11
ITAX	ITAL
ITA7	ITA2B
ITA6	ITA5
ITA9	ITA8
ITA3	ITAV
	ITA4
	ITAE

Clupeocephala (Actinopterygii)

b

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ITA3_P26006    - LLLWKCDFFKRTRYYQIMPKYHAVRIR...
ITA3_P26006_1  - LLLWKCGFFKRARTRALYEAKRQKAEM...

ITA6_P23229    - FILWKCGFFKRSRYDDSVPRYHAVRIR...
ITA6_P23229-3  - FILWKCGFFKRSRYDDSVPRYHAVRIR...
ITA6_P23229-5  - FILWKCGFFKRSRYDDSVPRYHAVRIR...
ITA6_P23229-2  - FILWKCGFFKRNKKDHYDATYHKAEIH...
ITA6_P23229-4  - FILWKCGFFKRNKKDHYDATYHKAEIH...
ITA6_P23229-6  - FILWKCGFFKRNKKDHYDATYHKAEIH...
ITA6_P23229-7  - FILWKCGFFKRNKKDHYDATYHKAEIH...
ITA6_P23229-9  - FILWKCGFFKRNKKDHYDATYHKAEIH...

ITA7_Q13683    - LLLWKMGFFKRAKHPEATVPQYHAVK...
ITA7_Q13683-3  - LLLWKMGFFKRAKHPEATVPQYHAVK...
ITA7_Q13683-7  - LLLWKMGFFKRAKHPEATVPQYHAVK...
ITA7_Q13683-9  - LLLWKMGFFKRAKHPEATVPQYHAVK...
ITA7_Q13683-13 - LLLWKMGFFKRAKHPEATVPQYHAVK...
ITA7_Q13683-10 - LLLWKCGFFHRSSQSSSFPTNYHRAC...
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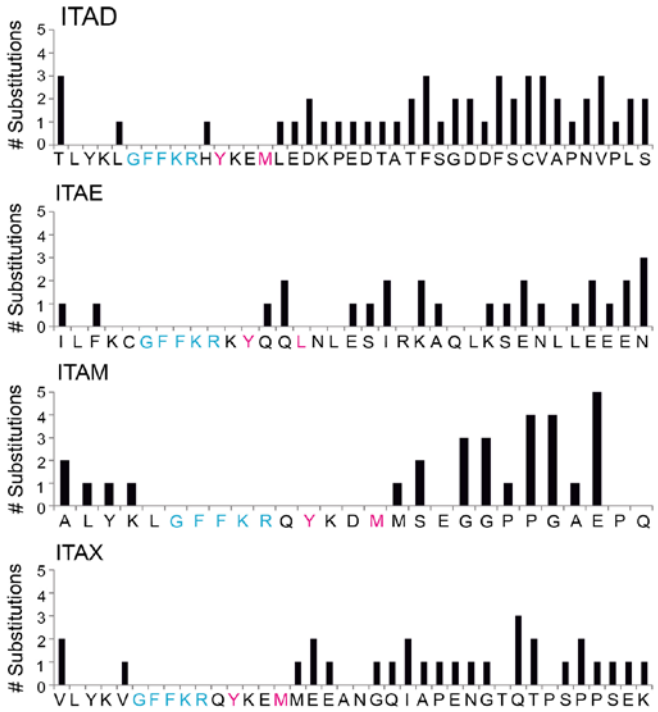
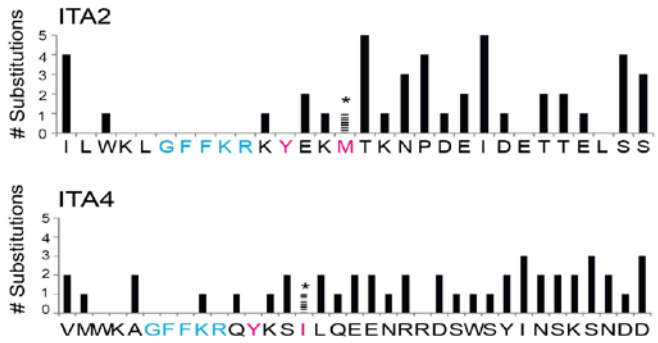
c

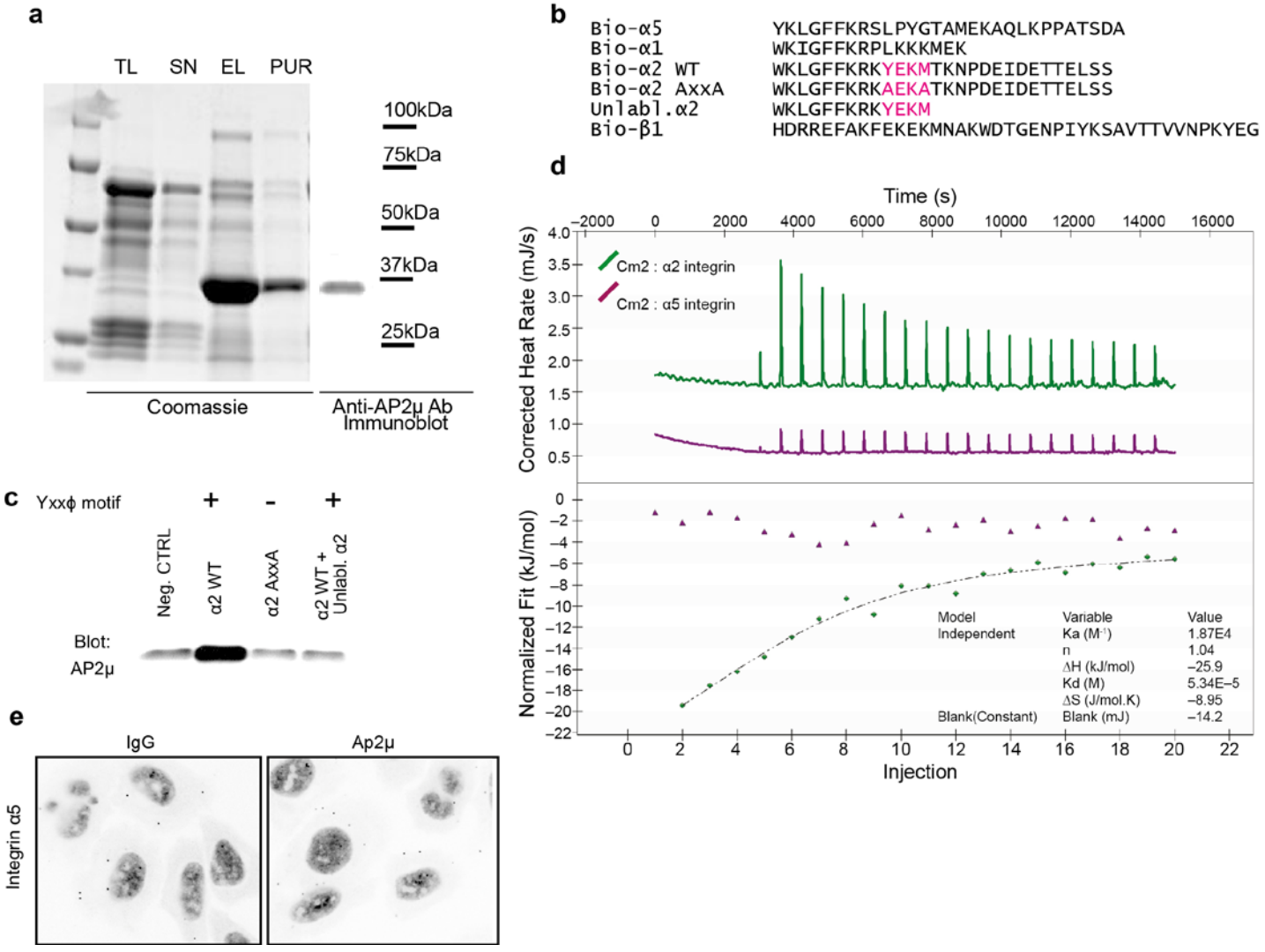


d



Supplementary Figure 1. Evolutionary and splicing analysis of Yxxϕ motif distribution

a: Comparison of the distribution of the Yxxϕ motif across integrin α-subunits in *Homo Sapiens* and *Clupeocephala*.
b: Alignment of membrane proximal and cytoplasmic region of splice variants of α3, α6 and α7 integrins from *Homo Sapiens*. Uniprot accession codes are indicated; the Yxxϕ motif is highlighted in red.
c: Residue variation across 6 mammals in membrane-proximal and cytoplasmic region of ITAD, ITAE, ITAM, ITAX (organisms: *Homo Sapiens, Mus Musculus, Canis Familiaris, Equus Caballus, Sus Scrofa, Bos Taurus*). Values on the Y-axis indicate the number of substitution for each residue.
d: Residue variation across 10 organisms in membrane-proximal and cytoplasmic region of ITA2 and ITA4 (*Homo Sapiens, Mus Musculus, Canis Familiaris, Equus Caballus, Sus Scrofa, Bos Taurus, Gallus Gallus, Xenopus Tropicalis, Anolis Carolinensis and Danio Rerio*). Asterisks indicate substitutions that are compatible with the Yxxϕ motif.



Supplementary Figure 2. Characterization of C-μ 2 interaction with α-integrins

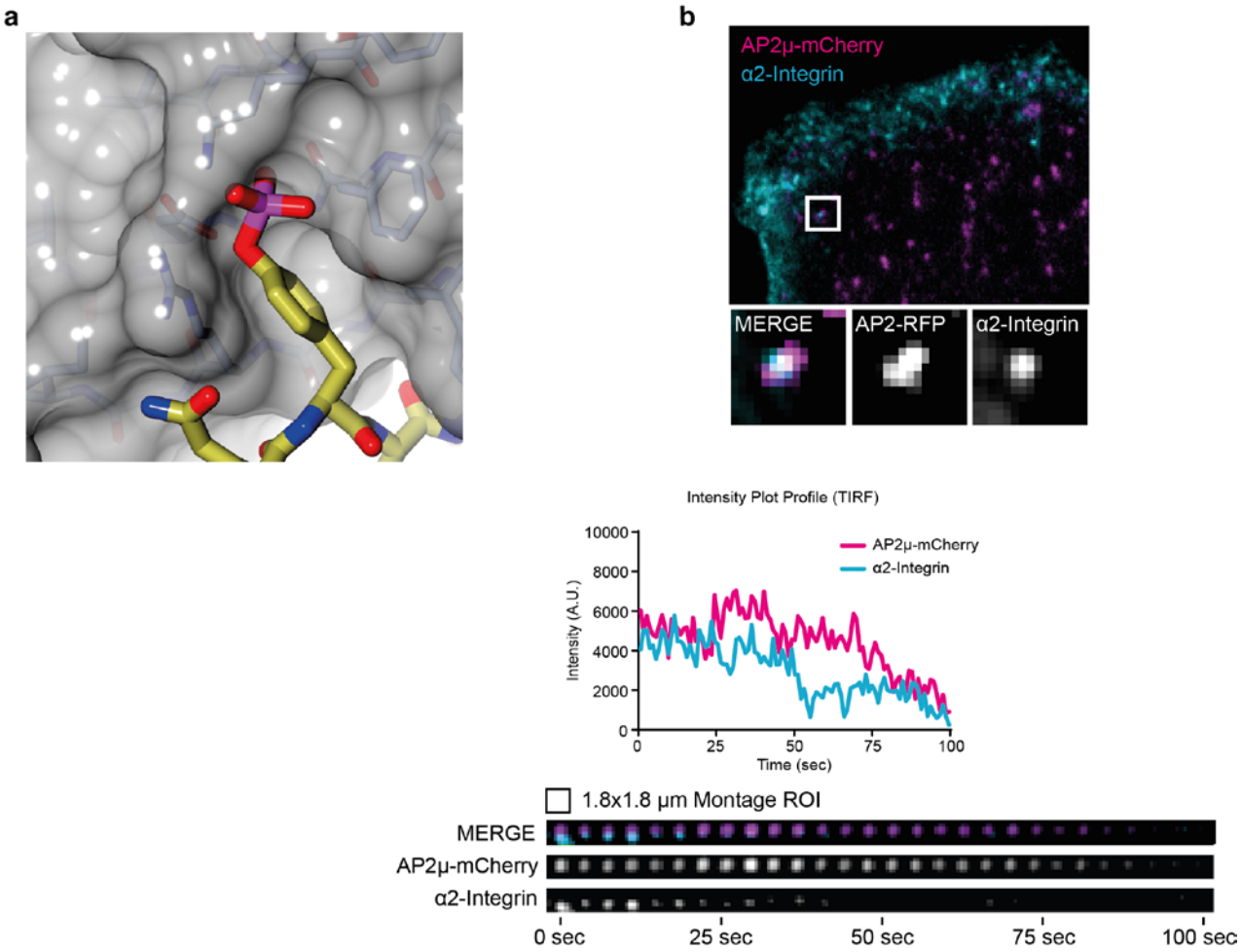
a: Purification of recombinant C-μ2. Enrichment of AP2μ in subsequent purification steps is shown, along with immunoblot with AP2μ Ab.

b: Sequences of the peptides used in this study.

c: Pulldown assay with recombinant C-μ2 and integrin biotinylated peptides. Equal amount of AP2μ and equimolar peptide concentrations were added to each sample. Neg. CTRL= beads alone. Tenfold excess of soluble, unlabelled α2 peptide was pre-incubated with AP2 μ CT.

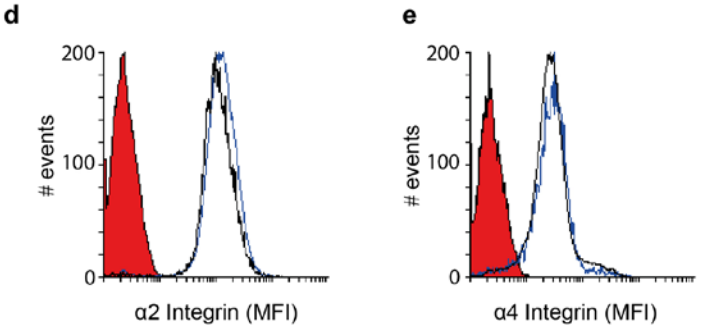
d: Representative isothermal titration calorimetry of integrin α2 peptide binding to C-μ2 (magenta). No detectable binding was seen for integrin α5 peptide (black).

e: Proximity Ligation assay between endogenous AP2 and endogenous α5 integrin.



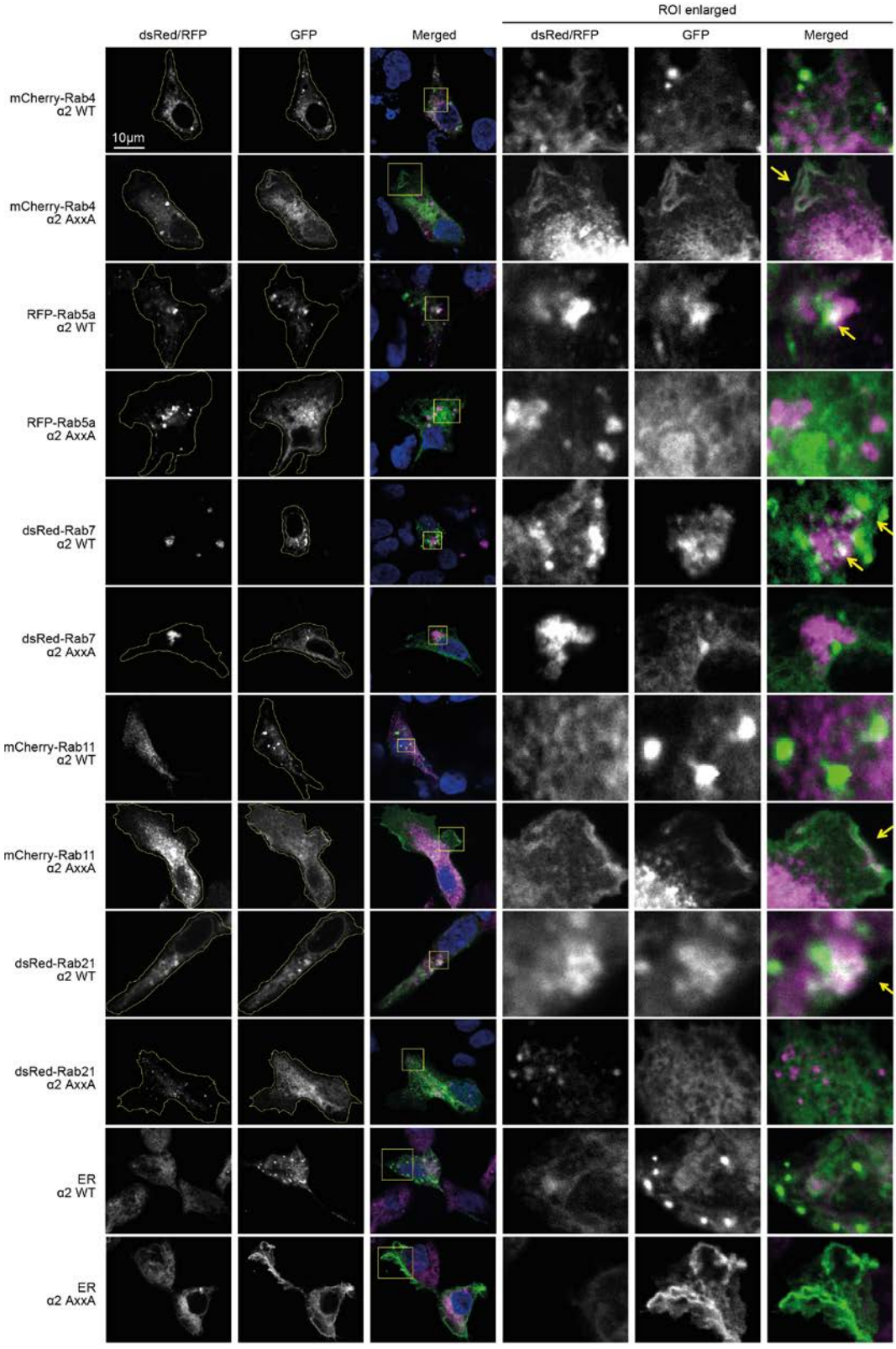
c

α2-GFP WT	WKLGFFKRKYEKMTKNPDEIDETTELSS
α2-GFP AxxA	WKLGFFKRKAEKATKNPDEIDETTELSS
α4-GFP WT	WKAGFFKRQYKSILQEENRRDSWSYINSKSNDD
α4-GFP AxxA	WKAGFFKRQAKSALQEENRRDSWSYINSKSNDD
αv-GFP WT	YRMGFFKRVRPPQEEQEREQLQPHENGEGNSET
αv-GFP (1)YERM	YRMGFFKRVYERMEEQEREQLQPHENGEGNSET
αv-GFP (1)AxxA	YRMGFFKRVAEKAEEQEREQLQPHENGEGNSET
αv-GFP (2)YERM	YRMGFFKRVRYERMQEEQEREQLQPHENGEGNSET
αv-GFP (2)AxxA	YRMGFFKRVRAEKAQEEQEREQLQPHENGEGNSET



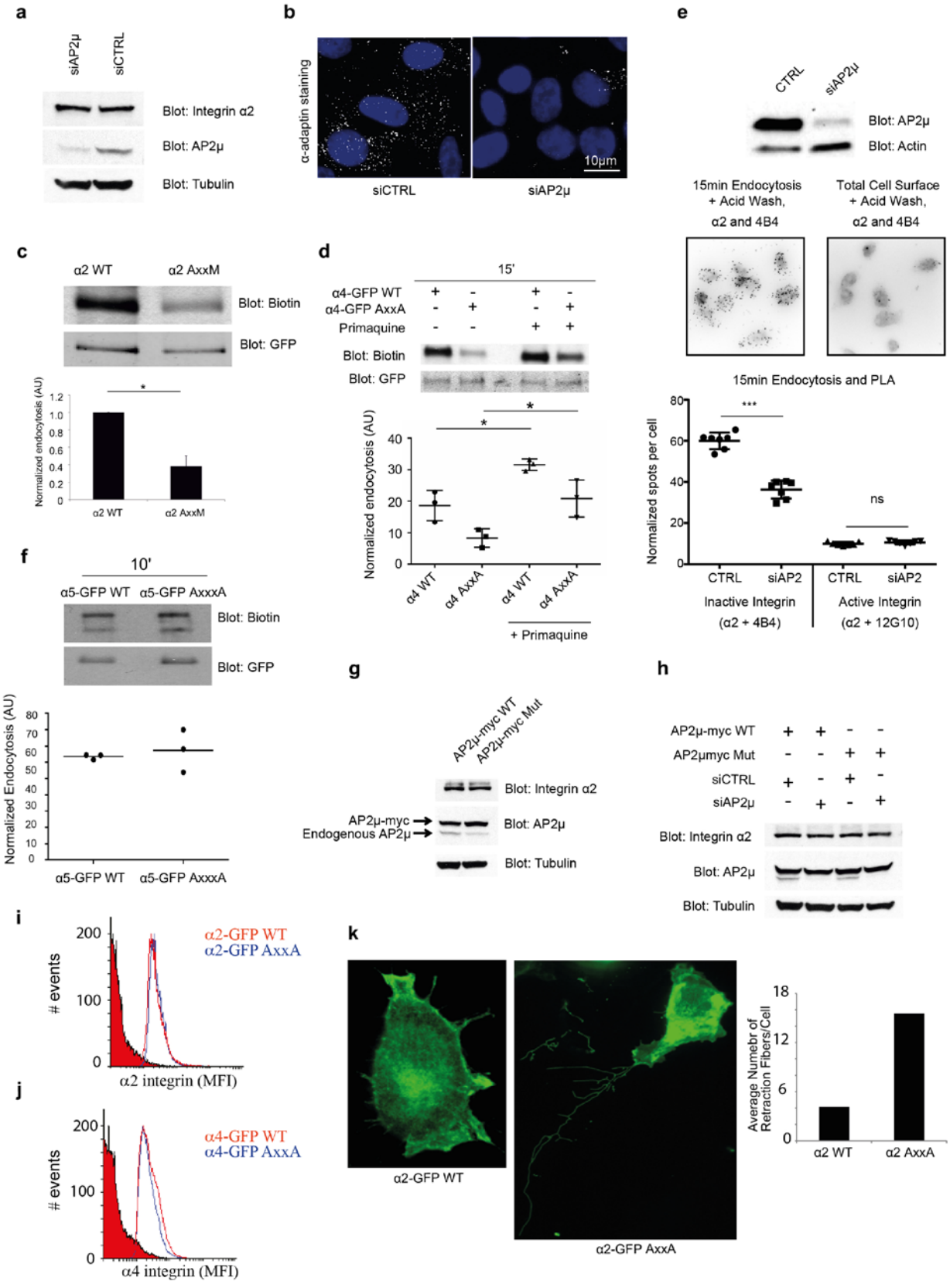
Supplementary Figure 3. Controls and additional information related to figures 2 and 3

a: Modelling of a tyrosine-phosphorylated integrin α4 peptide (yellow backbone) in the C-μ2 binding pocket. The phosphate head group of pY1009 (red) would not fit in the binding pocket.
b: Co-endocytosis of AP2μ and endogenous α2-integrin in MDA-MB-231 cells expressing AP2μ-mCherry labelled with α2-integrin antibody (MCA2025) during live cell imaging. TIRF plane is shown. Dynamics of AP2μ-RFP and endogenous α2-integrin co-endocytosis was measured with live cell TIRFM over a 100 sec period. Fluorescence intensities of a single AP2μ-positive pit were plotted over time.
c: Sequence of the membrane-proximal and cytoplasmic domains of GFP-tagged integrin constructs used in this study.
d: Surface α2 integrin levels detected by antibody labeling and fluorescence-activated cell sorting (FACS) analysis on HeLa cells. Solid red: Control IgG. Black line: GFP α2 WT cells stained with anti-α2 antibody; blue line: GFP α2 AxxA stained with anti-α2 antibody. GFP-positive cells were gated.
e: Surface α4 integrin levels detected by antibody labeling and fluorescence-activated cell sorting (FACS) analysis on HeLa cells. Solid red: Control IgG. Black line: GFP α4 WT stained with anti-α4 antibody; blue line: GFP α4 AxxA stained with anti-α4 antibody. GFP-positive cells were gated.


ROI enlarged
dsRed/RFP
GFP
Merged
dsRed/RFP
GFP
Merged
mCherry-Rab4 α2 WT
10μm
mCherry-Rab4 α2 AxxA
RFP-Rab5a α2 WT
RFP-Rab5a α2 AxxA
dsRed-Rab7 α2 WT
dsRed-Rab7 α2 AxxA
mCherry-Rab11 α2 WT
mCherry-Rab11 α2 AxxA
dsRed-Rab21 α2 WT
dsRed-Rab21 α2 AxxA
ER α2 WT
ER α2 AxxA

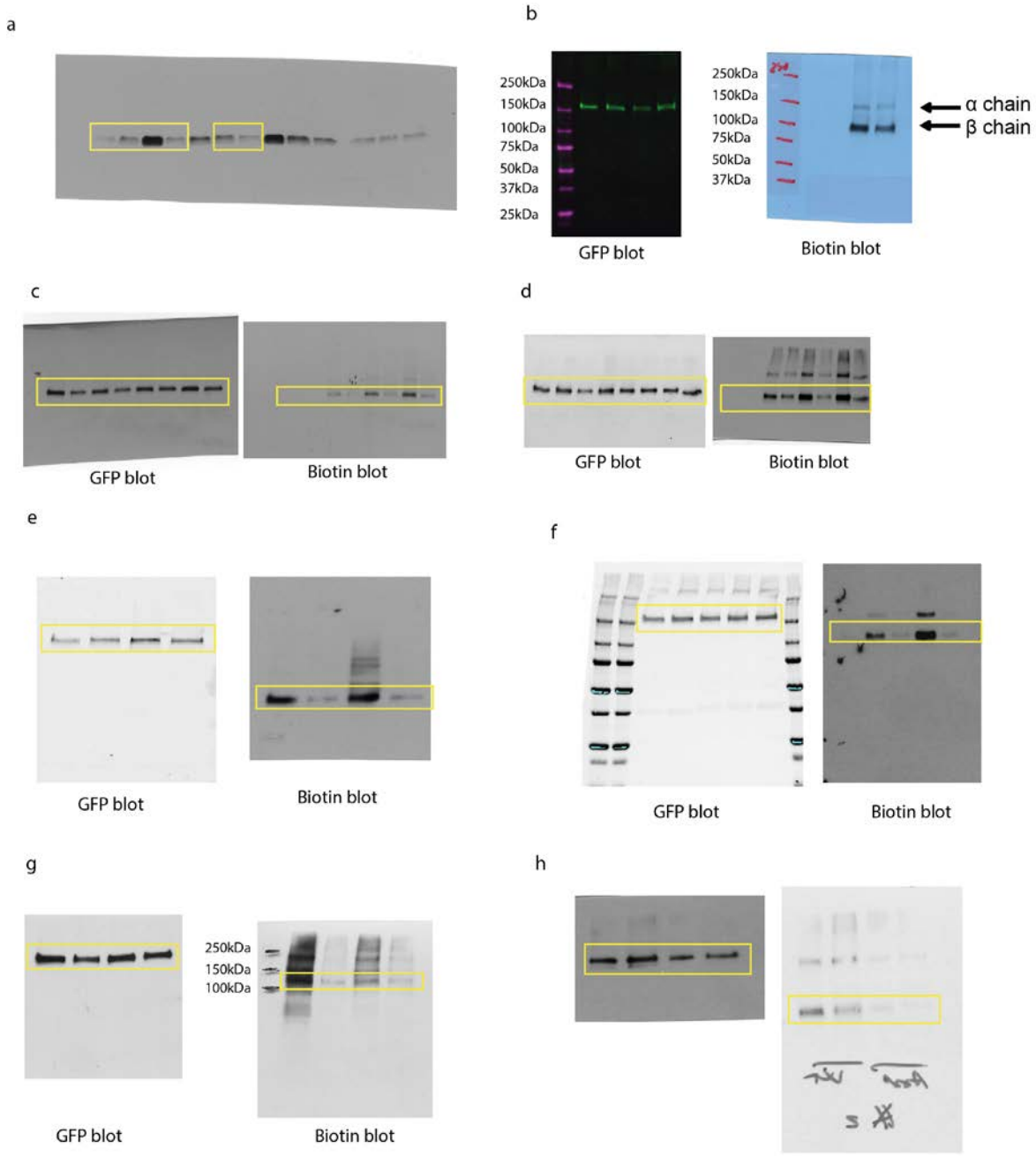
Supplementary Figure 4. Subcellular localization of α2-GFP WT or AxxA mutant.

Co-localization analysis of α2-GFP WT or AxxA mutant in HEK293 cells labelled with the ER-Tracker™ or overexpressing different Rab GTPases as indicated. Yellow boxes indicate the regions of interest (ROI) enlarged in the black and white images. Arrows point to areas of overlapping signal. Images are single planes from confocal stacks acquired with 63x magnification.


a
siAP2μ
siCTRL
Blot: Integrin α2
Blot: AP2μ
Blot: Tubulin
b
α-adaptin staining
10μm
siCTRL
siAP2μ
e
CTRL
siAP2μ
Blot: AP2μ
Blot: Actin
15min Endocytosis + Acid Wash, α2 and 4B4
Total Cell Surface + Acid Wash, α2 and 4B4
15min Endocytosis and PLA
Normalized spots per cell
***
ns
CTRL
siAP2
CTRL
siAP2
Inactive Integrin (α2 + 4B4)
Active Integrin (α2 + 12G10)
c
α2 WT
α2 AxxM
Blot: Biotin
Blot: GFP
Normalized endocytosis (AU)
*
α2 WT
α2 AxxM
d
15'
α4-GFP WT
α4-GFP AxxA
Primaquine
Blot: Biotin
Blot: GFP
Normalized endocytosis (AU)
*
*
α4 WT
α4 AxxA
α4 WT
α4 AxxA
+ Primaquine
f
10'
α5-GFP WT
α5-GFP AxxxA
Blot: Biotin
Blot: GFP
Normalized Endocytosis (AU)
α5-GFP WT
α5-GFP AxxxA
g
AP2μ-myc WT
AP2μ-myc Mut
Blot: Integrin α2
AP2μ-myc
Endogenous AP2μ
Blot: AP2μ
Blot: Tubulin
h
AP2μ-myc WT
AP2μmyc Mut
siCTRL
siAP2μ
Blot: Integrin α2
Blot: AP2μ
Blot: Tubulin
i
α2-GFP WT
α2-GFP AxxA
events
α2 integrin (MFI)
j
α4-GFP WT
α4-GFP AxxA
events
α4 integrin (MFI)
k
α2-GFP WT
α2-GFP AxxA
Average Numebr of Retraction Fibers/Cell
α2 WT
α2 AxxA

Supplementary Figure 5. Controls and additional information related to Figures 4 and 5

a: Western blot analysis of AP2µ, α2-integrin and tubulin levels in AP2µ and control siRNA transfected HeLa cells.
b: AP2µ or control silenced HeLa cells stained for endogenous AP2 α-adaptin (white) and dapi (blue).
c: Biotin-based endocytosis assays in HEK293 cells transfected with GFP-tagged α2-integrin WT or AxxM mutant. Biotin signal was normalized against total α2 amount measured from the GFP blot. Time point: 15 minutes (mean ± SEM; n = 3; *, $p<0.05$ (unpaired Student's t test; 2-tails distribution)).
d: Biotin-based endocytosis assays in HEK293 cells expressing either GFP-tagged α4-integrin WT or AxxA mutant in the presence or absence of primaquine treatment. Biotin signal was normalized against total α4 amount measured from the GFP blot. Time point: 15 minutes. Bars indicate (mean ± SEM; n=4 (n=biological replicates, each one being an independent cell culture); *, $p<0.05$ (unpaired Student's t test; 2-tails distribution).
e: Antibody-based endocytosis assay using PLA to detect the active or inactive α2β1 heterodimers in AP2µ silenced background. Endocytosis of α2 was allowed for 15 min after which cells were fixed, permeabilized and counterstained with active and inactive epitope recognizing anti-β1 antibodies (clones 12G10 and 4B4, respectively). The endocytosed PLA signal was normalized against total cell surface α2β1 levels (α2+4B4 for inactive and α2+12G10 for active heterodimers). Bars indicate mean±s.e.m.; n=2 (n=biological replicates, each one being an independent cell culture. In total, 120–144 cells were analyzed for each condition); ***, $p<0.0001$ (Mann Whitney test; 2-tails distribution).
f: Biotin-based endocytosis assays in HEK293 cells expressing GFP-tagged α5-integrin WT or AxxxA mutant. Endocytosis was allowed for 10 min and biotin signal was normalized against total α5 amount measured from the GFP blot. n=3 (n=biological replicates, each one being an independent cell culture).
g: Western blot analysis of AP2µ, α2-integrin and tubulin levels in HEK293 stably expressing AP2µ-myc WT or F174A/D176S mutant.
h: Western blot analysis of AP2µ, α2-integrin and tubulin levels in HEK293 stably expressing AP2µ-myc WT or F174A/D176S mutant and transfected with control or AP2µ siRNA.
i: Surface α2 integrin levels detected by antibody labeling and fluorescence-activated cell sorting (FACS) analysis on GD25b1A cells. Solid red: α2 Ab staining of non-transfected cells. Red line: α2-GFP WT-gated cells; blue line: α2-GFP AxxA-gated cells.
j: Surface α4 integrin levels detected by antibody labeling and fluorescence-activated cell sorting (FACS) analysis on GD25b1A cells. Solid red: α4 Ab staining of non-transfected cells. Red line: α4-GFP WT-gated cells; blue line: α4-GFP AxxA-gated cells.
k: Retraction fibers in HEK293 cells plated on collagen I and expressing either α2-GFP WT or AxxA mutant.



Supplementary Data Set 1. Uncropped blots

a: Uncropped blot of Fig.1d. Single band obtained by blotting with anti-AP2µ antibody: the single band corresponds to purified recombinant AP2µ, as shown in Supplementary Figure 2a.

b: Example of immunodetection of GFP and biotin, including the molecular marker used to quantify all the biotin-based endocytosis assays. The pattern of bands obtained in the context of this protocol is very peculiar and reproducible. The upper band (about 150kDa) corresponds to α2-GFP or α4-GFP as indicated. The lower band (just above 100kDa) corresponds to β1 integrin, which is co-immunoprecipitated with the paired α2-GFP or α4-GFP protein. The β1 band is shown and quantified for sake of clarity and to minimize the influence of the background on the quantification, since the signal from the β1 band is always stronger. It can be noticed, however, that the α and β band

intensities are always correlated.
c: Uncropped blots of Fig.4b.
d: Uncropped blots of Fig.4c.
e: Uncropped blots of Fig.4d.
f: Uncropped blots of Fig.5a.
g: Uncropped blots of Fig.5b.
h: Uncropped blots of Fig.5c.

Legends for Supplementary Movies

1. Video 1: AP2μ–integrin α2 coendocytosis event
   Single ROI of AP2μ positive clathrin pit is shown (2μm square). MDA-MB-231 cells were transfected with AP2μ-mCherry and endogenous α2-integrin was labelled using antibody (MCA2025). TIRF images were captured in 1s intervals. Movie is shown 20FPS.
2. Video 2: Localization and dynamics of α2-GFP WT
   Single plane of a HEK 293 cell plated on collagen I-coated glass-bottom dish. Localization and dynamics of α2-GFP WT-positive vesicles are shown.
3. Video 3: Localization and dynamics of α2-GFP AxxA
   Single plane of a HEK 293 cell plated on collagen I-coated glass-bottom dish. Membrane localization and dynamics of α2-GFP AxxA at lamellipodium are shown.
4. Video 4: HEK 293 cell expressing α2-GFP WT spreading on collagen I
   Bottom plane of a HEK 293 cell expressing α2-GFP WT spreading on Collagen I. α2-GFPpositive vesicles and dynamic tubular compartments are visible.
5. Video 5: HEK 293 cell expressing α2-GFP AxxA spreading on collagen I
   Bottom plane of a HEK 293 cell expressing α2-GFP AxxA spreading on Collagen I. Plasma membrane localization of α2-GFP is shown.