Supporting Information

The large G protein alpha-subunit XLαs limits clathrin-mediated endocytosis and regulates tissue iron levels in vivo

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Supplementary Table S1
Proteins identified by the preliminary proteomic screen selectively from purified lysates of C3H10T1/2 cells transduced with adenovirus encoding FLAG-tagged mouse XLαs; control cells were transduced with adenovirus encoding yellow fluorescent protein.

Gene Accession	**Protein**	**Function**
Gnas_IPI:IPI00119853.1	XLαs	The Bait
Gnas_IPI:IPI00471141.2	ALEX	Unknown (interacts with XLαs)
Gnb1_IPI:IPI00120716.3	Gbeta subunit 1	G protein subunit
Gnb1_IPI:IPI00120716.3	Gbeta subunit 3	G protein subunit
Gng10_IPI:IPI00110583.1	Ggamma subunit 10	G protein subunit
Snx9_IPI:IPI00313275.7	Sorting nexin 9	Endocytosis
Dnm1_IPI:IPI00272878.6	Dynamin-1	Endocytosis
Dnm2_IPI:IPI00131445.2	Dynamin-2	Endocytosis

Supplementary Table S2

sgRNA targeting sequences used to knockout mouse XLαs in this study.

No.	Spacer Sequence (5'-3')	Reverse Complement(5'-3')
1	CTACTCCGCGGCCTCCTCCG	CGGAGGAGGCCGCGGAGTAG
2	TTGCTGACCCGCCTACTCCG	CGGAGTAGGCGGGTCAGCAA
3	TGGCCTGCCCCGGAGCCACA	TGTGGCTCCGGGGCAGGCCA

Supplementary Table S3

DNA sequences of wild-type and mutant clones. The guide sequences are marked in yellow. Deletions are shown as dotted line, and insertion is show in red.

sgRNA1

WT TGCTGACCCGCCTACTCCGCGGCCTCCTCCGCGGCCGACTGCCTGG

KO1 TGCTGACCCGCCTACTCCGCGGCCTCCTCCGC--CCGACTGCCTGG

TGCTGACCCGCCTACTCCGCGGCCTCCGTGCG---CGACTGCCTGG

sgRNA2

WT AGGTTGCTGACCCGCCTACTCCGCGGCCTCCTCCGCGCGGCCGACTG

KO2 AGGTTGCTGAC----------TG

AGGTTGCTGACCCGCCTA-----CGGCCTCCTCCGCGCGGCCGACTG

KO3 AGGTTGCTGACCCGCCTA-TCCGCGGCCTCCTCCGCGCGGCCGACTG

AGGTTGCTGACCCGCCTACT----------TCCGCGCGGCCGACTG

sgRNA3

WT CGAAACCGCCCTGGCCTGCCCCGGAGCCACACGGTCGGGAGCAAC

KO4 CGAAACCGCCCTGGCCTGCCCCGGAGCC-CACGGTCGGGAGCAAC

CGAAACCGCCCTGGCCTGCCCCG----CACACGGTCGGGAGCAAC

Supplementary Figure Legends:

Supplementary Figure S1. XLαs can be co-immunoprecipitated with SNX9 and Dnm2. Skeletal muscle tissue lysates from P0 WT mice were immunoprecipitated with anti-XLαs, anti-Dnm2, or anti-SNX9 antibodies, and then, immunoblot was performed using XLαs antiserum. Goat IgG and the rabbit IgG served as negative controls for immunoprecipitation. Skeletal muscle tissue lysates from P0 WT and XLKO mice were used for immunoblotting to identify the specific XLαs band.

Supplementary Figure S2. Sequence traces obtained from the different XLαs knockout clonal cells. (A) KO1, (B) KO2, (C) KO3 and (D) KO4. Blue arrows indicate the locations of the introduced mutations; note that each Gnas allele in a clonal cell is mutated differently, leading to two overlapping sequence traces.

Supplementary Figure S3. Ablation of XLαs induces transferrin/iron uptake. (A) WT or XLKO Ocy454 cells were untreated or treated with transferrin-Alexa Fluor 568 for 30 min, and then analyzed by flow cytometry to determine the amount of transferrin uptake. Gated events are shown as overlaid histograms of transferrin-Alexa Fluor 568 fluorescence (x-axis) against the number of events (y-axis). (B) Primary cardiomyocytes from P2 WT and XLKO pups were untreated or treated with transferrin-Alexa Fluor 568 for 30 min, and then analyzed by flow cytometry. (C) Iron overload-induced apoptosis is increased in the absence of XLαs. Primary cardiomyocytes from WT and KO mice were untreated or treated with $FeCl_3$ for 72 hours, followed by annexin V/PI flow cytometry assay for determining apoptotic cell population.

Supplementary Figure S4. Overexpression of XLαs represses SNX9 expression. (A) After transfection with XLαs-GFP, GFP- and GFP+ cell populations were selected via flow cytometry. (B) Sorted GFP- and GFP+ cells were immunoblotted with SNX9 antibody. (C) Densitometric analysis of the relative abundances of SNX9 in GFP- and GFP+ cells. Data are mean ± SEM of two independent experiments (n=6 per group). **, $p<0.01$.

Supplementary Figure S5. Suppression of SNX9 rescues enhanced transferrin uptake in XLKO Ocy454 cells. WT and XLKO Ocy454 cells were transfected with (A) control siRNA, or (B) SNX9 siRNA. siRNA-transfected Ocy454 cells were untreated or treated with transferrin-Alexa Fluor 568 for 30 min, and then analyzed by flow cytometry for transferrin uptake.

Supplementary Figure S6. Overexpression of XLαs prevents internalization of PTHR. PTHR was co-transfected with (A) Gsα-GFP, or (B) XLαs-GFP in HEK293T cells. The cells were treated for 5 min with PTH-TMR (10nM), which results in receptor internalization. (C, D) A line intensity profile across the cell was obtained in a given image. Representative intensity profiles are shown in the right.

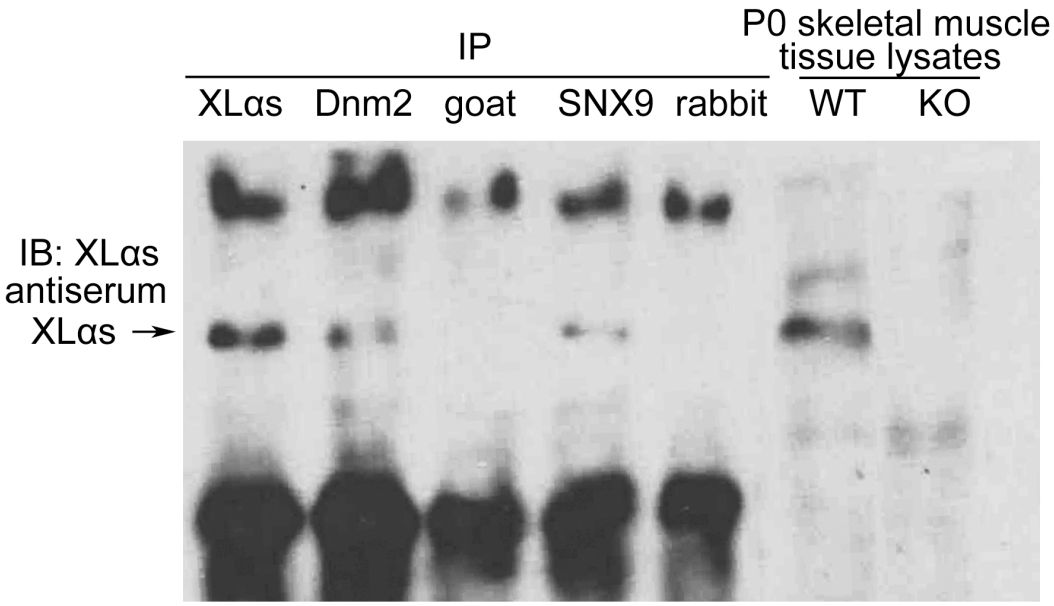
Supplementary Figure S7. TIRF microscopy analysis detecting PTHR on the cell surface in XLαs-GFP or Gsα-GFP overexpressed cells. HEK293T transfected with PTHR-dsRed and (A) Gsα-GFP, or (B) XLαs-GFP were monitored for green and red fluorescent signals in live cells by TIRF microscopy for 50 min after PTH(1-34) (10 nM) stimulation. Quantification of fluorescence intensities in (C) Gsα- and (D) XLαs-overexpressing cells. Data are mean ± SEM of 8 different cells from three independent experiments.

Supplementary Figure S8. HEK293T cells co-transfected with plasmids encoding V2R and either Gsα-GFP or XLαs-GFP were treated with VP-TMR (1 μM) for 5 min. Confocal analysis of (A) Gsα-GFP, or (B) XLαs-GFP transfected HEK293T cells. (C, D) Fluorescence intensities along the white lines in A and B were plotted as intensity histograms. (E) Relative ratios of fluorescence intensity between plasma membrane and cytosolic area of TMR and GFP were quantified by ImageJ by dividing the plasma membrane intensity to the cytosolic fluorescence intensity. Data are mean ± SEM of from ten different cells. *$P < 0.05$.

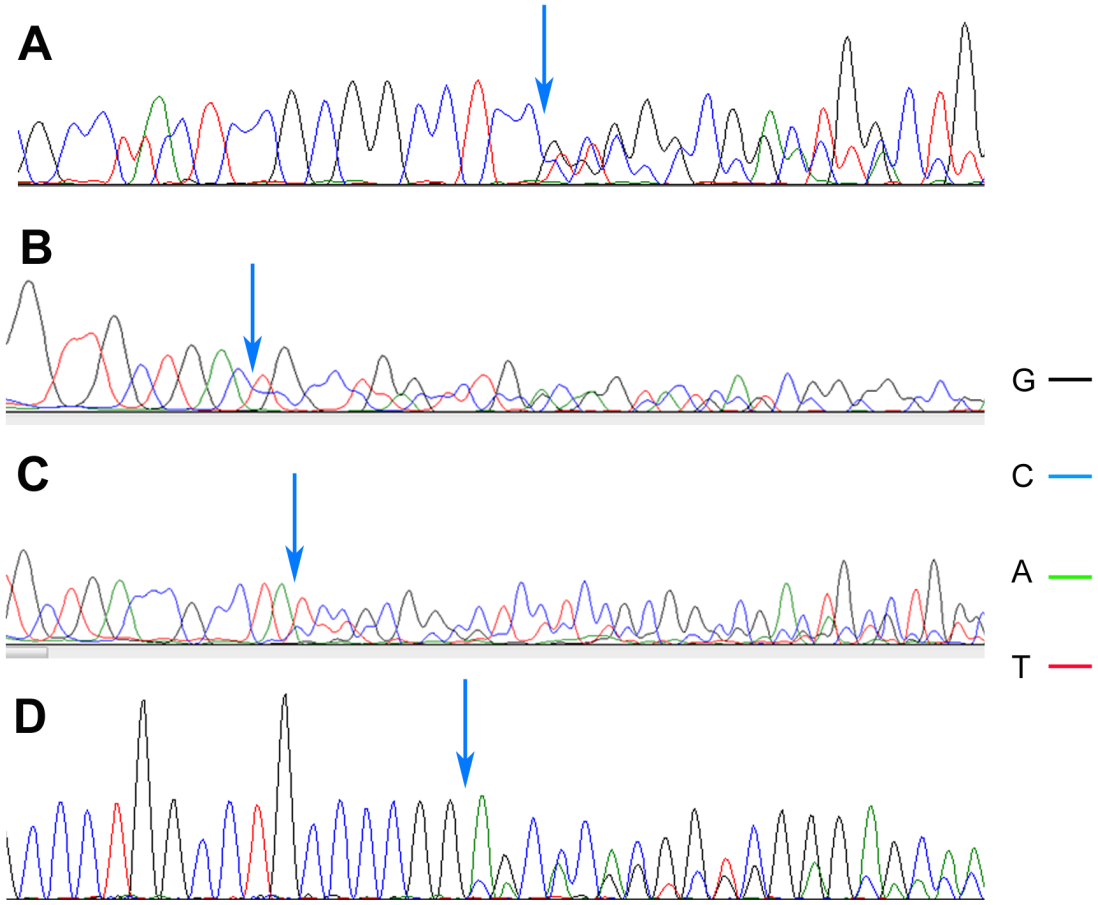
Supplementary Figure S9. Ablation of XLαs enhances PTHR internalization. Primary cardiomyocyte from WT and XLKO mice were transfected with PTHR plasmid, and subsequently treated with PTH-TMR for 1 min (A, B), 5 min (E, F), 15 min (G, H), and 30 min (J, K). Scale bar, 10μm. (C) Quantification of fluorescence intensities of cytosol and total cell in WT and KO cells after 1 min PTH-TMR treatment. Yellow line outlines outside of the cell, and blue line marks the estimated cytosolic region. (D) Relative ratio of fluorescence intensity between cytosolic and total cell area of PTHR was quantified by dividing the cytosolic intensity to the total intensity, using ImageJ. Data are mean ± SEM from fifteen different cells. (I) Percentage of cells with perinuclear PTH-TMR signal in WT and KO cells after 15 min treatment. 48-65 cells were randomly selected per group for quantification. Data are mean ± SEM from three independent experiments. *, $p<0.05$; **, $p<0.01$.

Supplementary Figure S10. ALEX(-)XLαs mutant inhibits transferrin uptake and PTHR internalization. (A) Confocal microscopy analysis of ALEX(-)XLαs-GFP expressing cells incubated with transferrin-Alexa Fluor 568 for 30 min. (B) Confocal microscopy analysis of PTHR internalization in ALEX(-)XLαs-GFP expressing cells. (C) Representative plots on transferrin uptake in ALEX(-)XLαs-GFP transfected HEK293T cells. (D) Quantification of the median values of transferrin-Alexa Fluor 568 in GFP+ and GFP- cell populations. Data are mean ± SEM of three independent experiments (n=9 per group). *, $p<0.05$.

Supplementary Fig. S1



Supplementary Fig. S2



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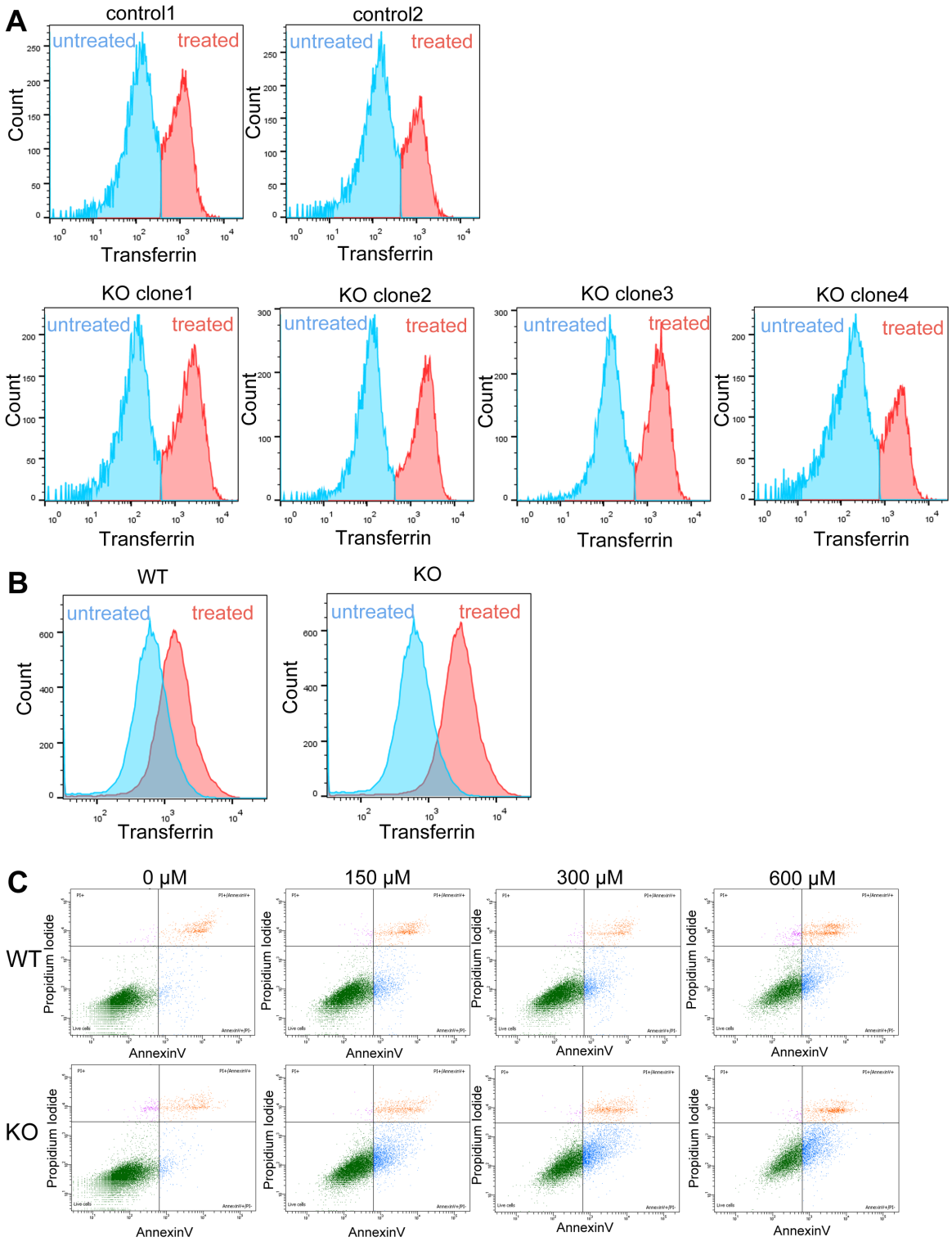

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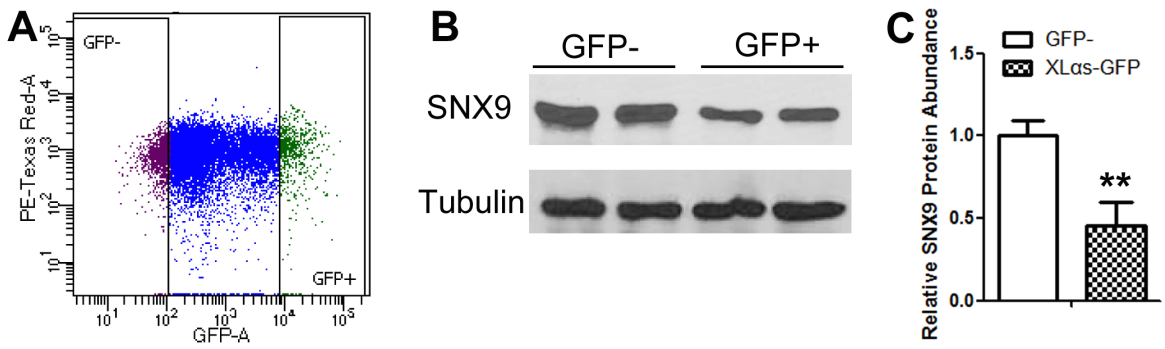

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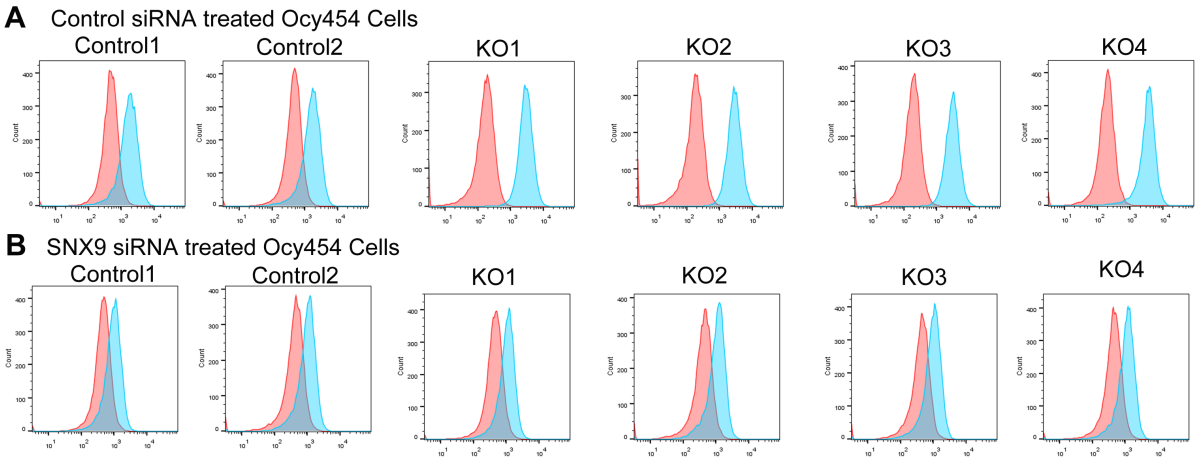

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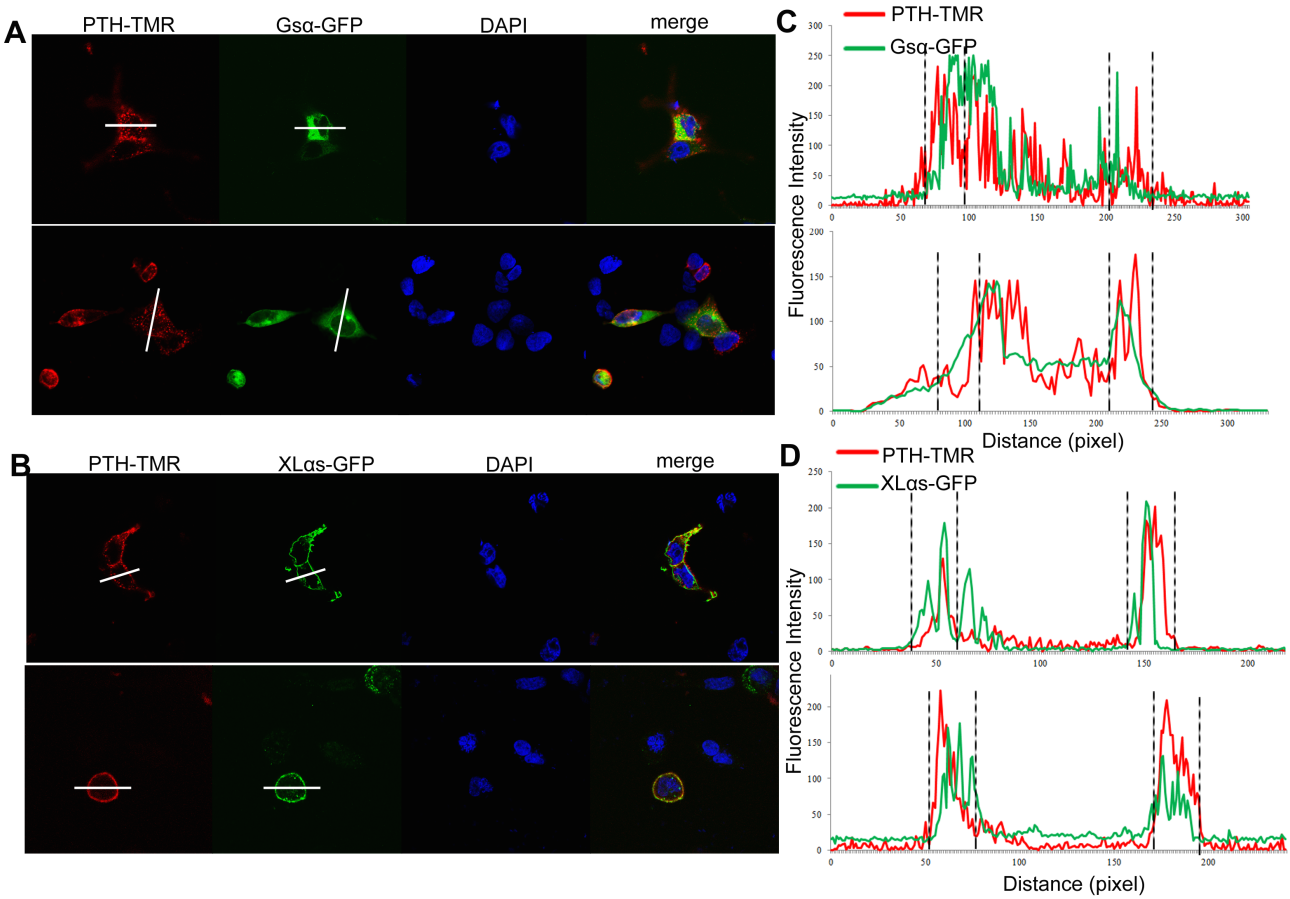

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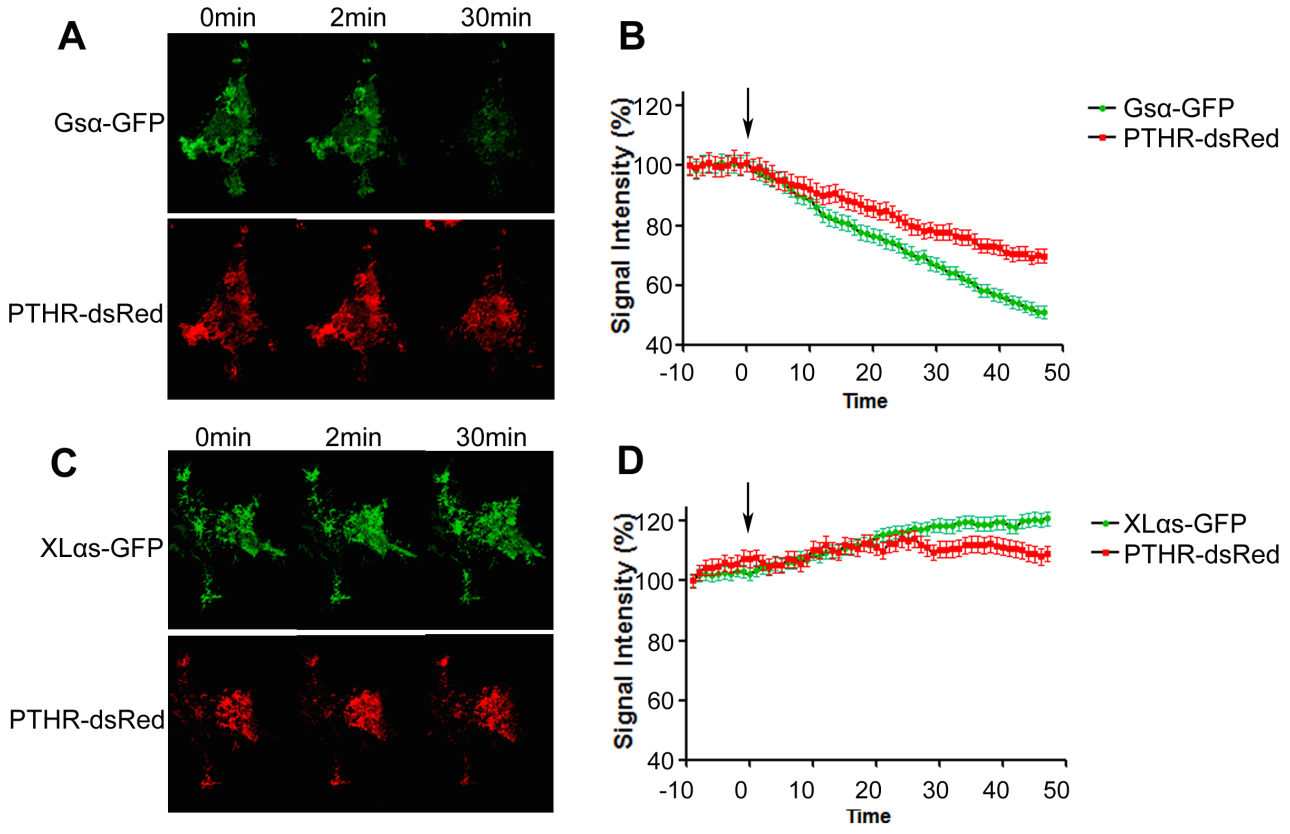

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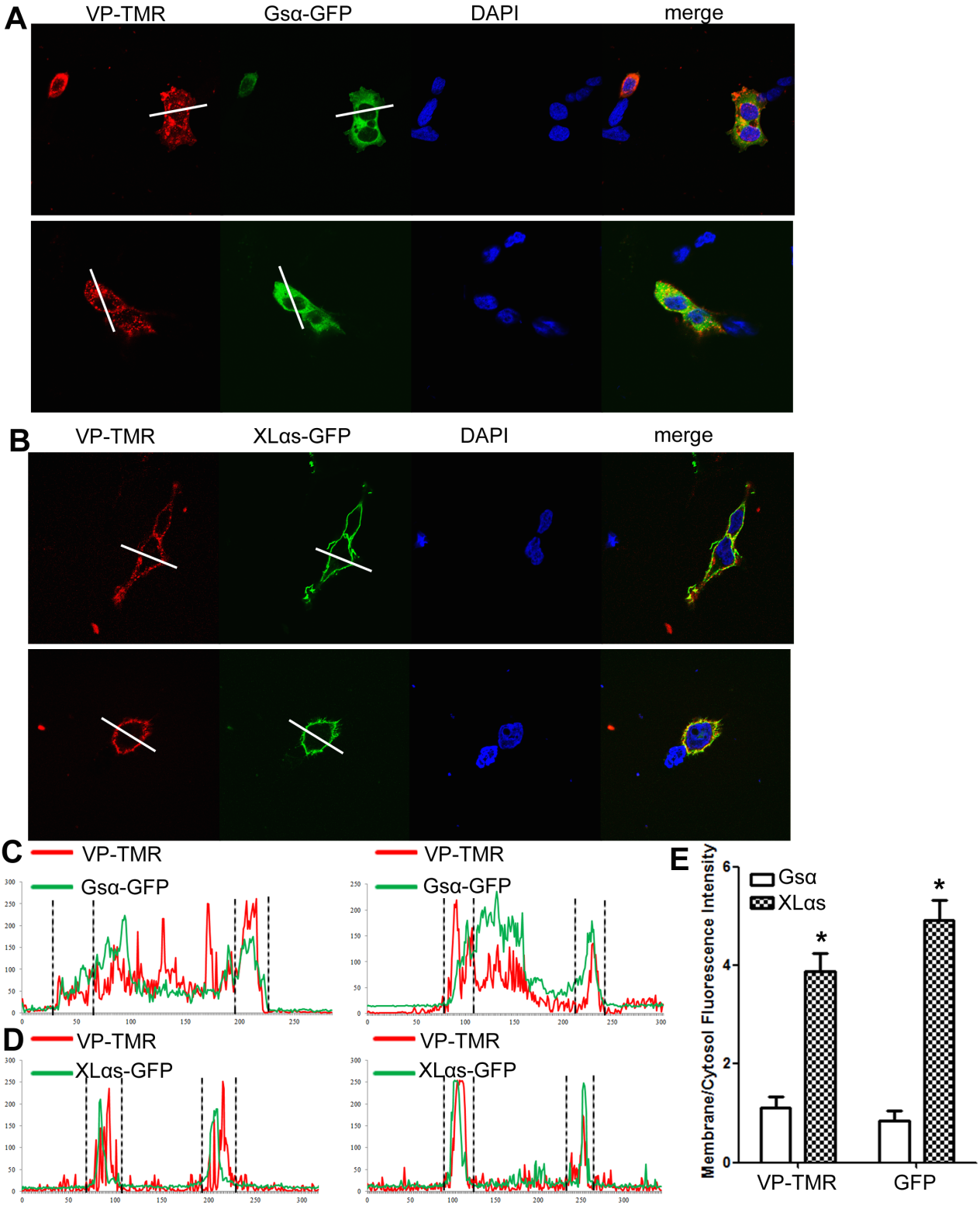

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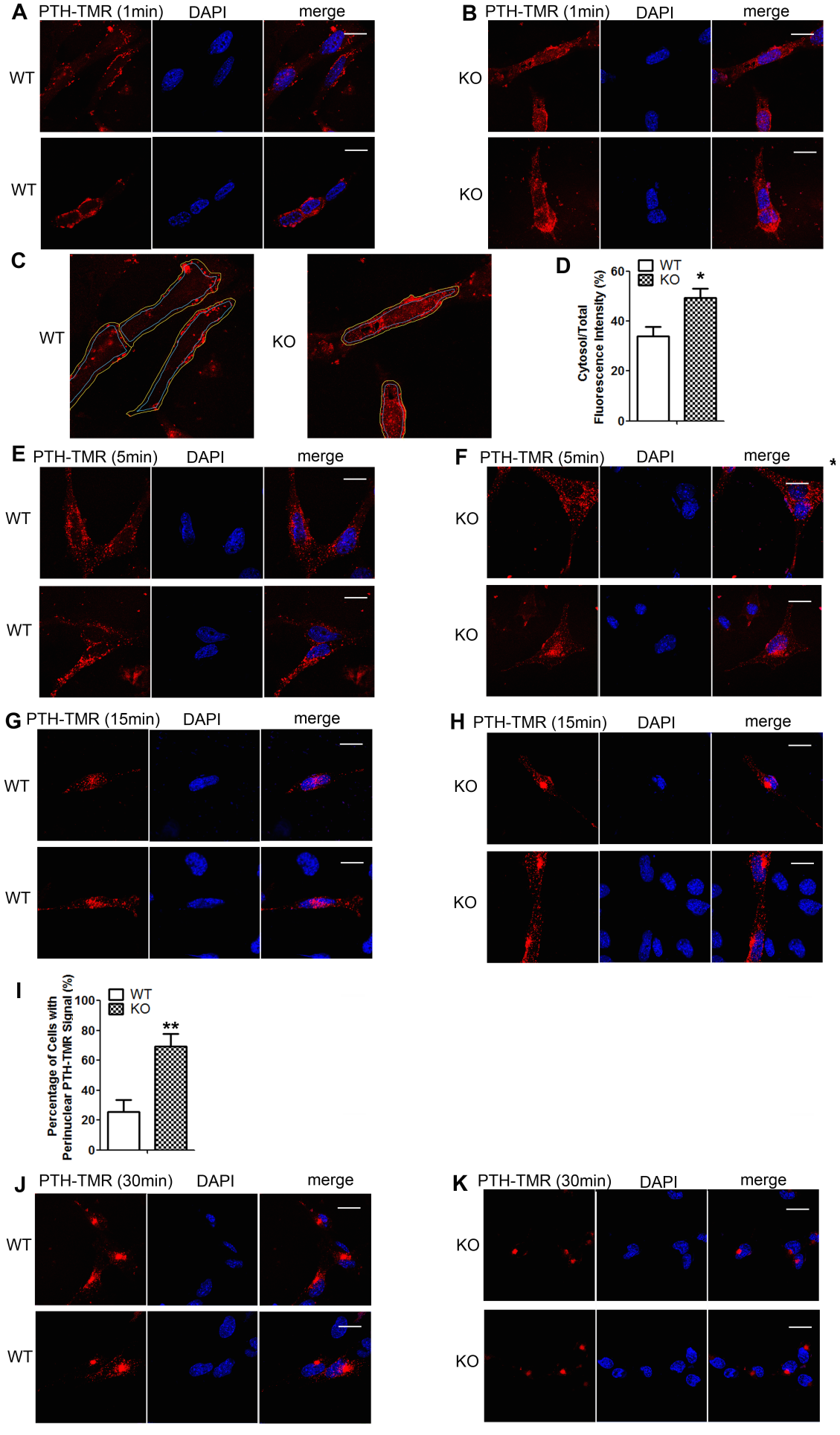

Supplementary Fig. S10