Supplementary Materials for

Homeostasis imbalance of YY2 and YY1 promotes tumor growth by manipulating ferroptosis

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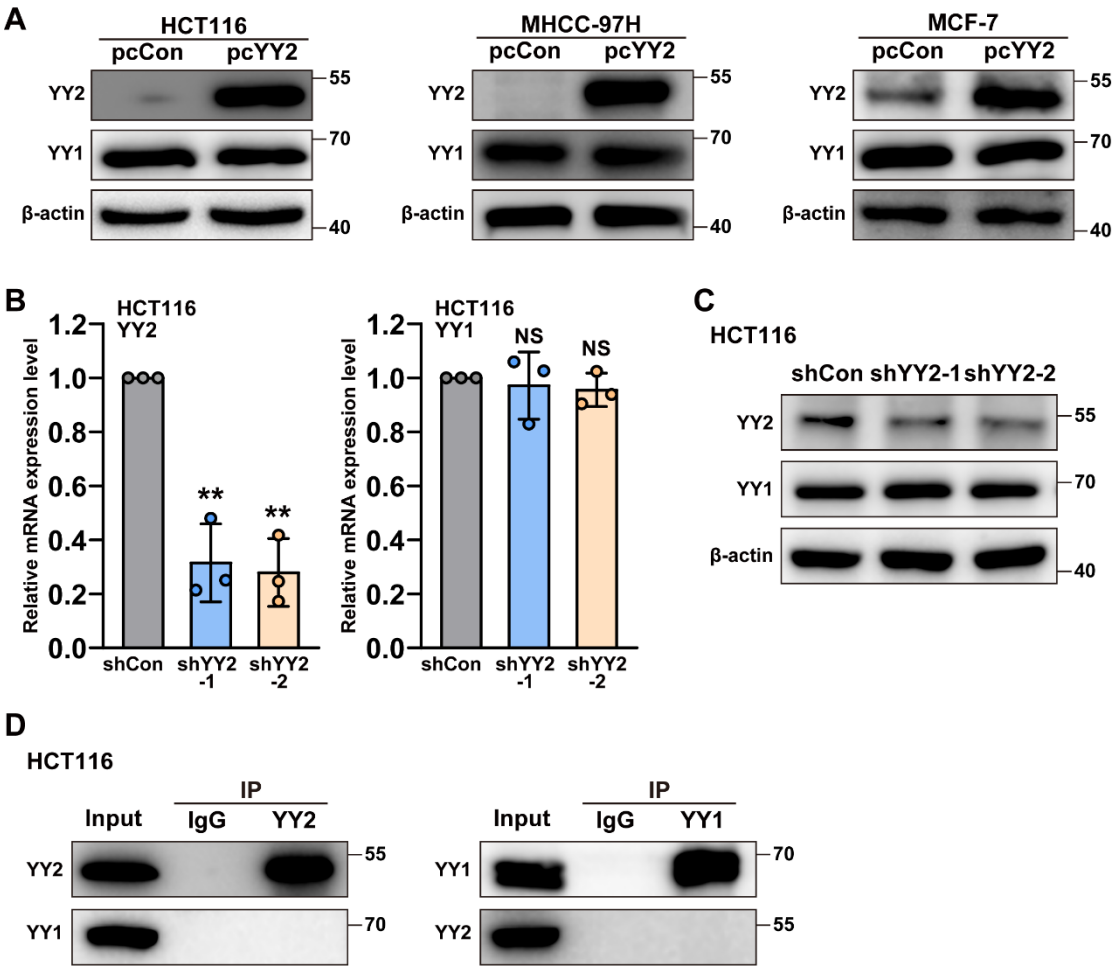
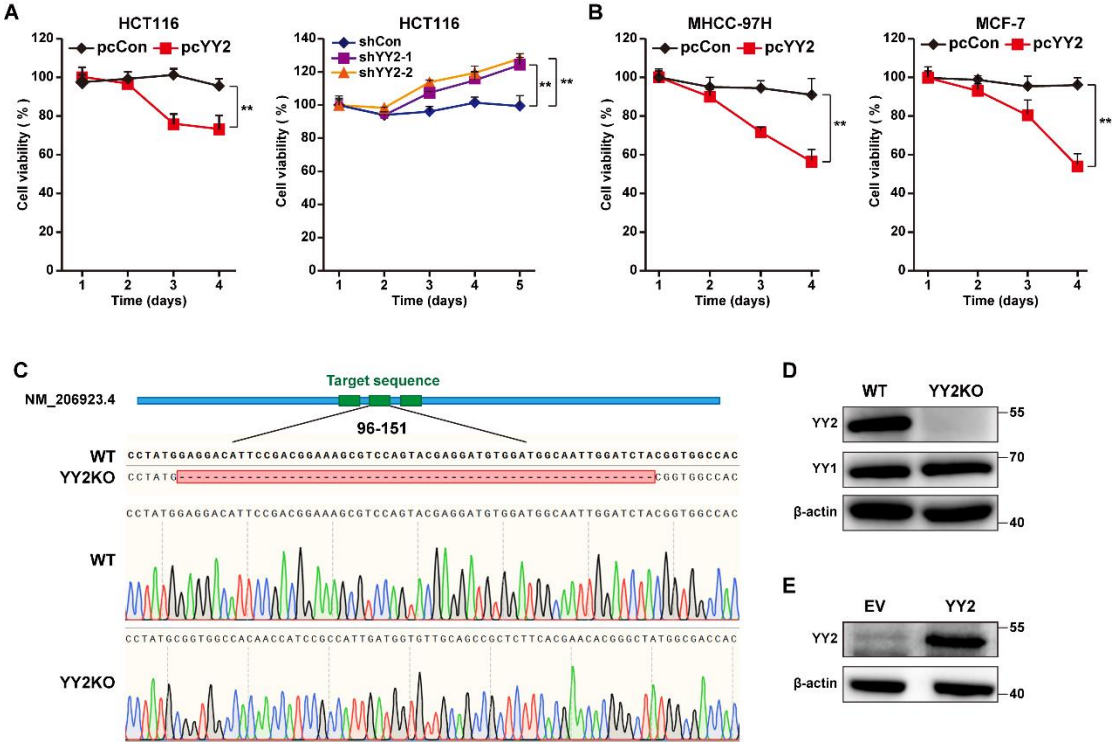
This PDF file includes:

Figure S1.



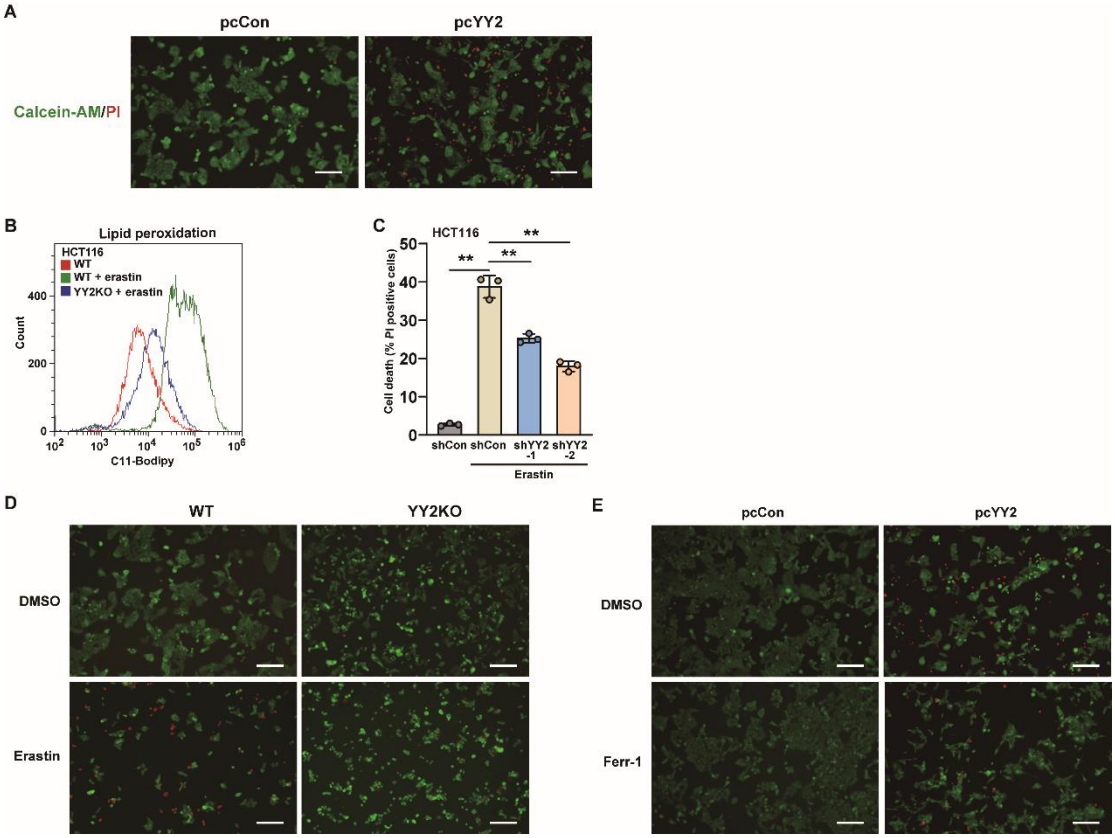
Supplementary Fig. S1. The efficacy and specificity of *YY2* overexpression vector, shRNA expression vectors targeting *YY2*, and antibodies used. (A) YY2 protein expression level in HCT116, MHCC-97H, and MCF-7 cells transfected with *YY2* overexpression vector, as determined using western blotting. **(B and C)** YY2 and YY1 mRNA (B) and protein (C) expression levels in HCT116 cells transfected with shRNAs targeting *YY2*, as determined using quantitative reverse-transcription PCR (qRT-PCR) and western blotting, respectively. **(D)** Specificity of the YY2 and YY1 antibodies used in this work, as determined using immunoprecipitation assay. Cells transfected with shCon or pcCon were used as controls. β-actin was used for qRT-PCR normalization and as western blotting loading control. Quantification data are shown as mean ± SD (n = 3). *P* values were calculated using two-tailed unpaired Student's t-test. pcCon: pcDNA3.1(+); ***P* < 0.01.

Figure S2.



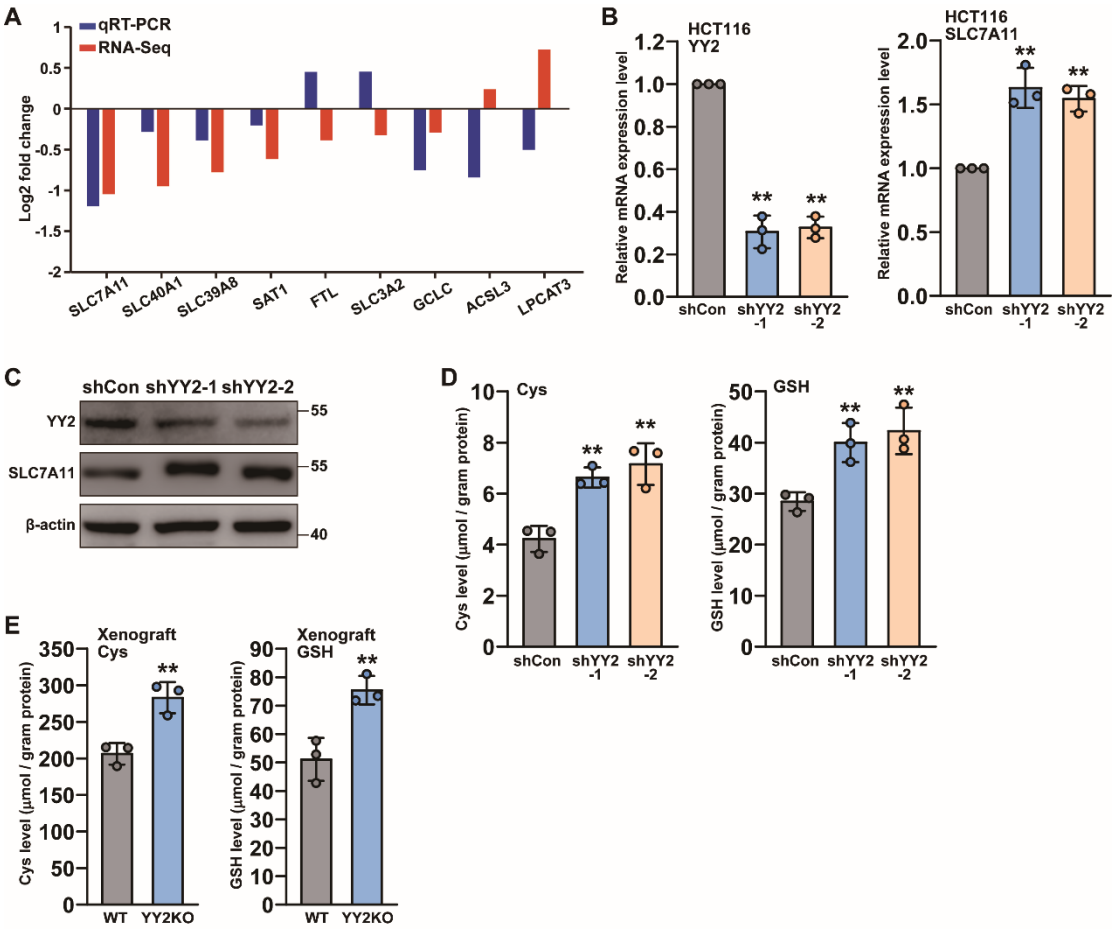
Supplementary Fig. S2. YY2 suppresses tumor cells viability. (A) Viabilities of *YY2*-overexpressed (left panel) and *YY2*-knocked down (right panel) HCT116 cells were measured at indicated time points. **(B)** Viabilities of MHCC-97H and MCF-7 cells overexpressing *YY2* were measured at indicated time points. **(C)** Establishment of *YY2*-knocked out HCT116 (HCT116^{YY2null}) cells. Sequencing results depicting the deleted region in HCT116^{YY2null} cells were shown. **(D)** YY2 and YY1 protein expression levels in HCT116^{YY2null} cells, as determined using western blotting. **(E)** YY2 protein expression level in HCT116 cells stably overexpressing *YY2* established using lentivirus, as determined using western blotting. Wild-type HCT116 cells, HCT116 cells transfected with shCon or pcCon, or infected with empty lentivirus (EV) were used as controls. β-actin was used for western blotting loading control. Quantification data are shown as mean ± SD (n = 3). *P* values were calculated using two-tailed unpaired Student's t-test. pcCon: pcDNA3.1(+); YY2KO: HCT116^{YY2null} cells; $^{**}P < 0.01$.

Figure S3.



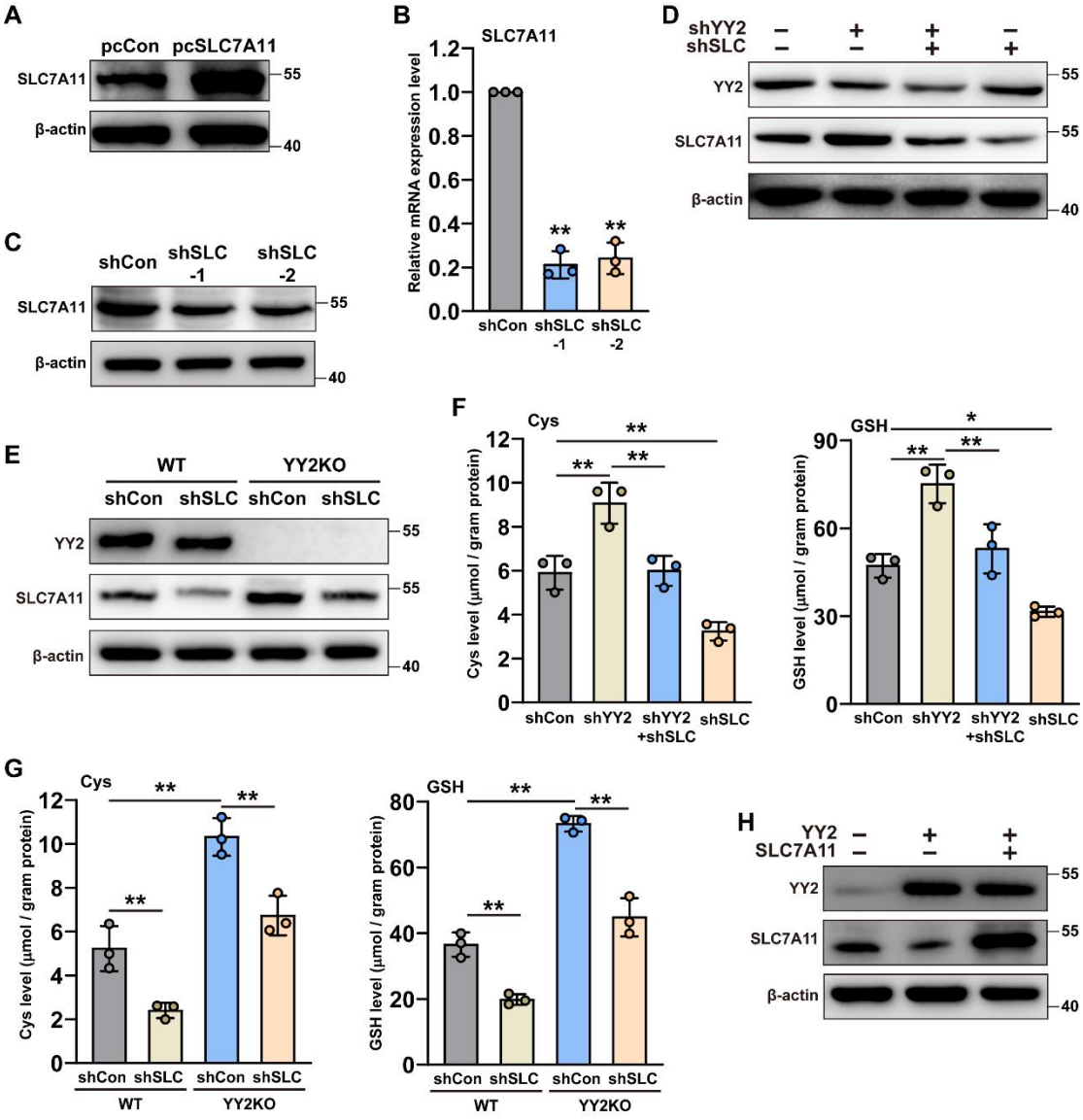
Supplementary Fig. S3. YY2 regulates tumor cells ferroptotic cell death. (A) Staining of living cells (calcein-AM, green) and dead cells (PI, red) in HCT116 cells overexpressing *YY2*. Scale bars, 100 μm. **(B)** Lipid peroxidation level in HCT116^{YY2null} cells treated with 20 μM erastin for 24 h, as assessed by C11-BODIPY staining and flow cytometry. **(C)** Cell death percentage of *YY2*-knocked down HCT116 cells treated with 20 μM erastin for 24 h, as determined using PI staining and flow cytometry. **(D)** Staining of living and dead cells in HCT116^{YY2null} cells treated with 20 μM erastin for 24 h using calcein-AM/PI. Scale bars, 100 μm. **(E)** Staining of living and dead cells in HCT116 cells overexpressing *YY2* and treated with 30 μM ferrostatin-1 for 24 h using calcein-AMP/PI. Scale bars, 100 μm. Wild-type HCT116 cells or HCT116 cells transfected with shCon or pcCon were used as controls. Quantification data are shown as mean ± SD (n = 3). *P* values were calculated using two-tailed unpaired Student's t-test. One-way ANOVA analyses were performed when more than two groups were compared. pcCon: pcDNA3.1(+); YY2KO: HCT116^{YY2null} cells; Ferr-1: ferrostatin 1; $^{**}P < 0.01$.

Figure S4.



Supplementary Fig. S4. *YY2*-silencing suppresses SLC7A11 and induces cellular cysteine and GSH levels. (A) Fold-change of mRNA expression levels of candidate target genes related to ferroptosis in HCT116 overexpressing *YY2* compared to HCT116 cells transfected with pcCon, as determined using qRT-PCR. **(B and C)** YY2 and SLC7A11 mRNA (B) and protein (C) expression levels in *YY2*-knocked down HCT116 cells, as determined using qRT-PCR and western blotting, respectively. **(D)** Cysteine (left) and GSH (right) levels in *YY2*-knocked down HCT116 cells. **(E)** Cysteine (left) and GSH (right) levels in xenografted tumors formed by wild-type HCT116 and HCT116^{YY2null} cells. Wild-type HCT116 cells or HCT116 cells transfected with shCon or pcCon were used as controls. β-actin was used for qRT-PCR normalization and as western blotting loading control. Quantification data are shown as mean ± SD (n = 3). *P* values were calculated using two-tailed unpaired Student's t-test. pcCon: pcDNA3.1(+); YY2KO: HCT116^{YY2null} cells; $^{**}P < 0.01$.

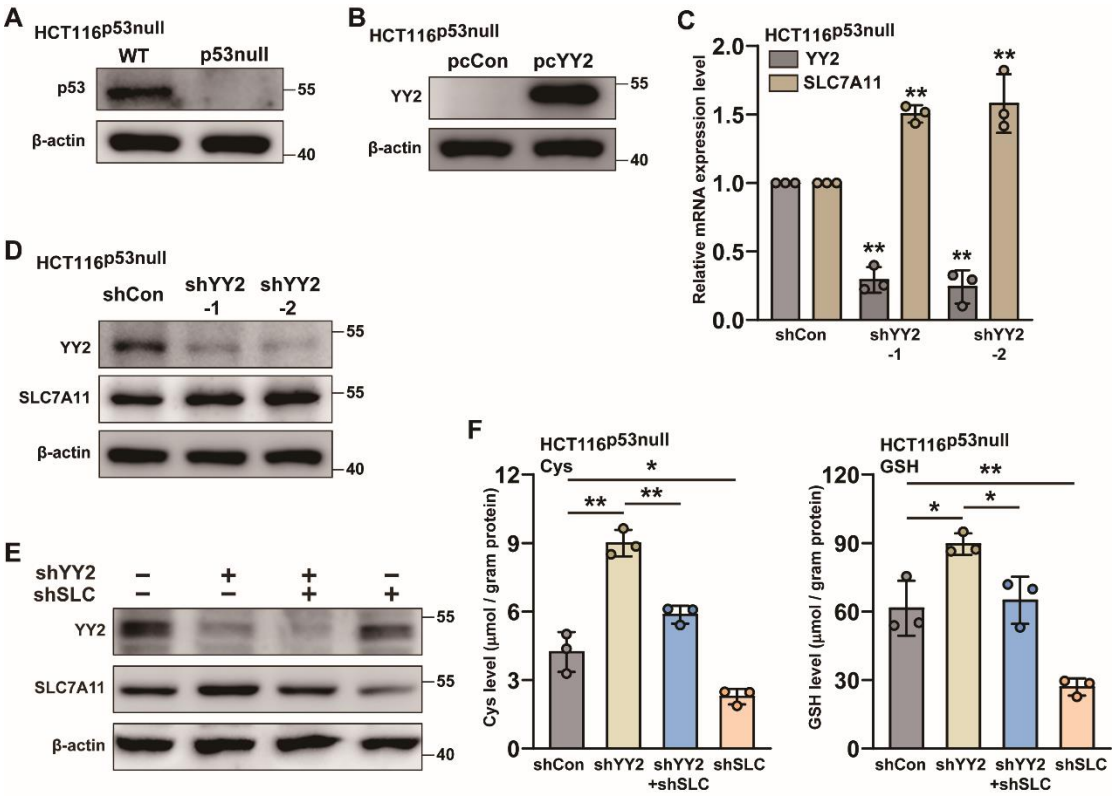
Figure S5.



Supplementary Fig. S5. SLC7A11 is crucial for YY2 suppression on cellular cysteine and GSH levels. (A) SLC7A11 protein expression level in HCT116 cells transfected with *SLC7A11* overexpression vector, as determined using western blotting. **(B and C)** SLC7A11 mRNA (B) and protein (C) expression levels in HCT116 cells transfected with shRNAs targeting *SLC7A11*, as determined using qRT-PCR and western blotting, respectively. **(D and E)** YY2 and SLC7A11 protein expression levels in *YY2*/*SLC7A11*-double knocked down (D) HCT116 and *SLC7A11*-knocked down HCT116^{YY2null} cells, as examined using western blotting. **(F and G)** Cysteine (left) and GSH (right) levels in *YY2*/*SLC7A11*-double knocked down HCT116 (F) and *SLC7A11*-knocked down HCT116^{YY2null} cells (G). **(H)** YY2 and

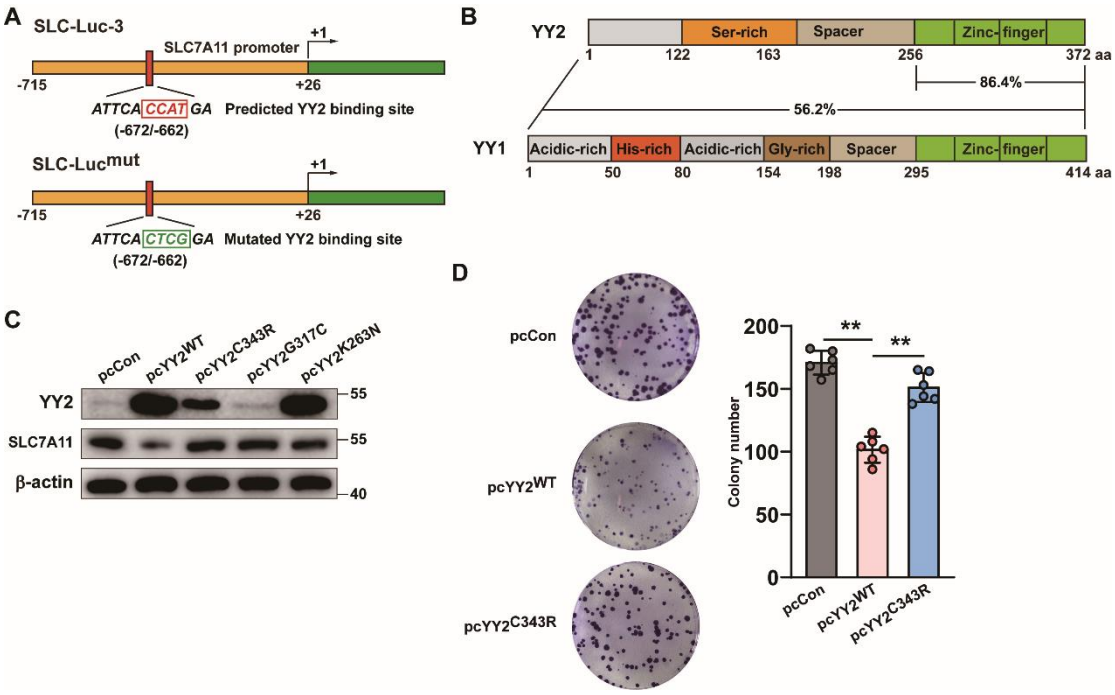
SLC7A11 protein expression levels in HCT116 cells infected with Lenti-YY2 (YY2) and Lenti-SLC7A11 (SLC7A11), as determined using western blotting. Wild-type HCT116 cells, HCT116 cells transfected with shCon or pcCon, or infected with empty lentivirus were used as controls. β-actin was used for qRT-PCR normalization and as western blotting loading control. Quantification data are shown as mean ± SD (n = 3). *P* values were calculated using two-tailed unpaired Student's t-test. One-way ANOVA analyses were performed when more than two groups were compared. pcCon: pcDNA3.1(+); YY2KO: $HCT116^{p53null}$ cells; **P* < 0.05; ***P* < 0.01.

Figure S6.



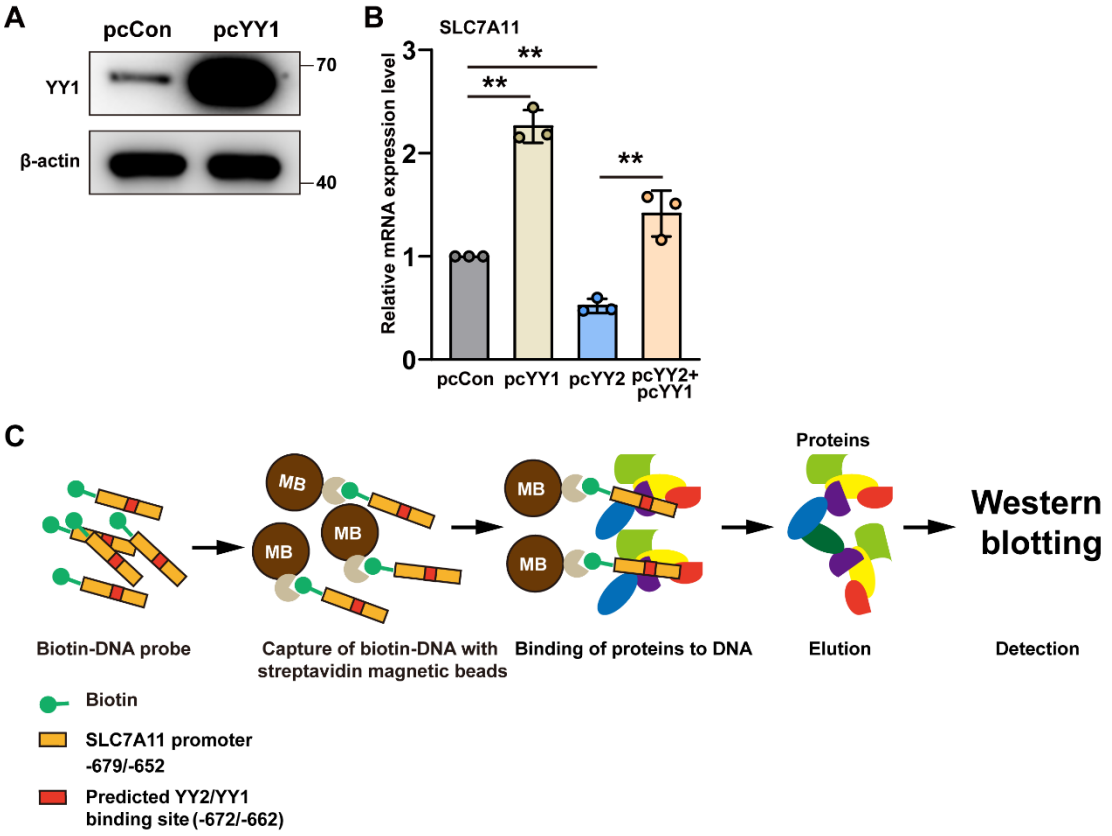
Supplementary Fig. S6. YY2 regulates SLC7A11/GSH synthesis axis in a p53-independent manner. (A) p53 protein expression level in HCT116^{p53null} cells, as determined using western blotting. **(B)** YY2 protein expression level in HCT116 ^{p53null} cells transfected with *YY2* overexpression vector, as determined using western blotting. **(C and D)** YY2 and SLC7A11 mRNA (C) and protein (D) expression levels in *YY2*-knocked down HCT116^{p53null} cells, as determined using qRT-PCR and western blotting, respectively. **(E)** YY2 and SLC7A11 protein expression levels in *YY2*/*SLC7A11*-double knocked down HCT116^{p53null} cells, as examined using western blotting. **(F)** Cysteine (left) and GSH (right) levels in *YY2*/*SLC7A11*-double knocked down HCT116^{p53null} cells. Cells transfected with shCon or pcCon were used as controls. β-actin was used for qRT-PCR normalization and as western blotting loading control. Quantification data are shown as mean ± SD (n = 3). *P* values were calculated using two-tailed unpaired Student's t-test. One-way ANOVA analyses were performed when more than two groups were compared. pcCon: pcDNA3.1(+); **P* < 0.05; ***P* < 0.01.

Figure S7.



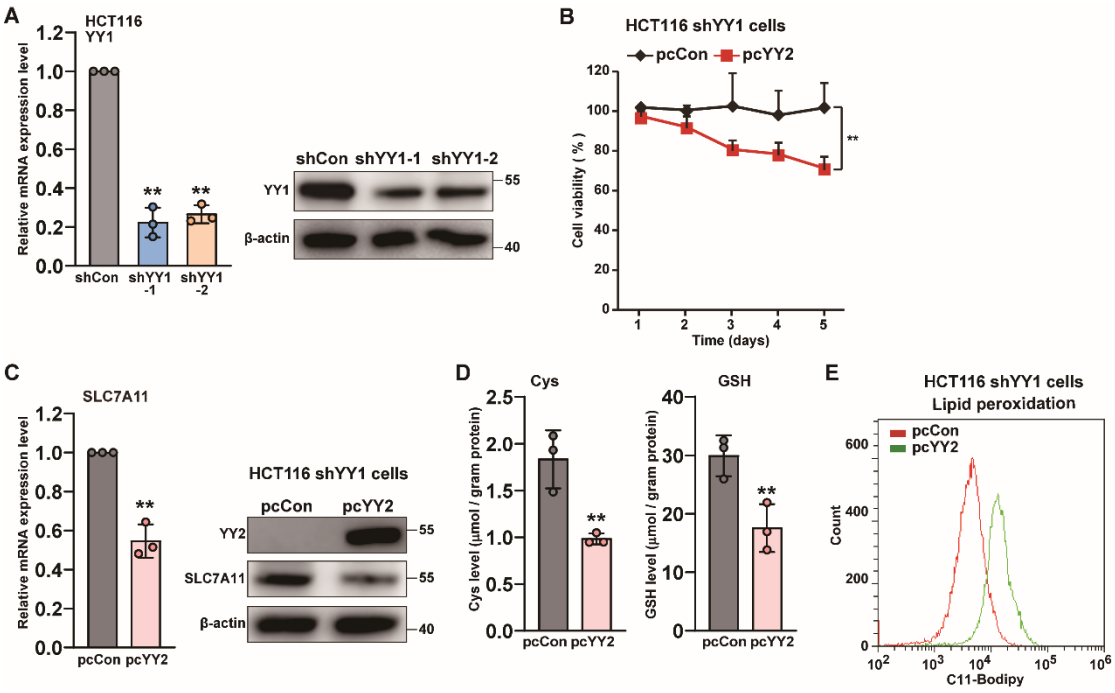
Supplementary Fig. S7. Mutation in zinc finger domains abolished YY2 regulatory effect on SLC7A11 expression. (A) Schematic diagram of reporter vector bringing the -715 to +26 region of *SLC7A11*-promoter (SLC-Luc-3; upper panel) and reporter vector bringing the -715 to +26 region of *SLC7A11*-promoter with mutated YY2 predicted binding site (SLC-Luc^{mut}; lower panel). The mutated nucleotides are shown in red. **(B)** Schematic diagram showing the comparison between the protein structures of YY2 and YY1. Percentages of identity between the whole proteins and the zinc-fingers domains are shown. **(C)** SLC7A11 protein expression level in HCT116 cells overexpressing cancer-associated *YY2* mutants and wild-type *YY2* (WT), as determined using western blotting. **(D)** Colony formation potential of HCT116 cells overexpressing *YY2*^{C343R} mutant. Representative images and quantification results (n = 6) are show. Cells transfected with pcCon or wild-type *YY2* overexpression vector were used as controls. β-actin was used for western blotting loading control. Quantification data are shown as mean ± SD. *P* values were calculated using one-way ANOVA analyses. pcCon: pcDNA3.1(+); $^{**}P < 0.01$.

Figure S8.



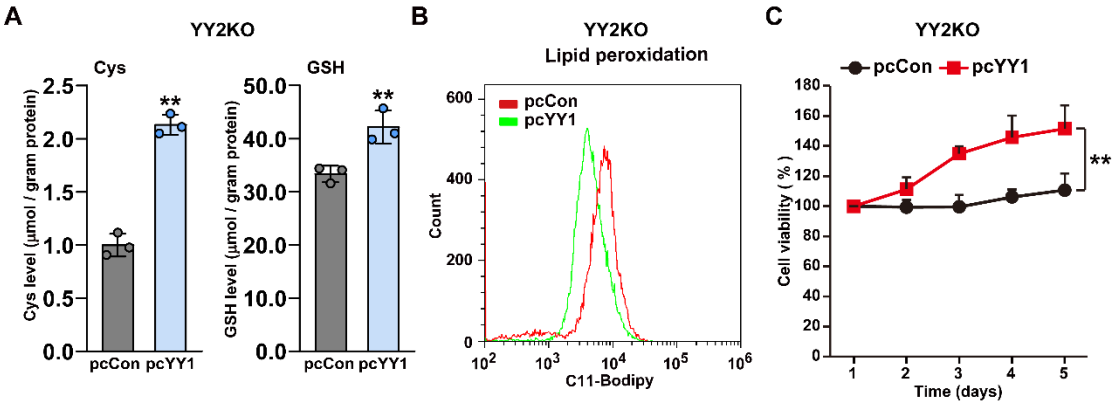
Supplementary Fig. S8. YY2 and YY1 regulate SLC7A11 expression level. (A) YY1 protein level in HCT116 cells transfected with pcYY1, as determined by western blotting. **(B)** SLC7A11 mRNA expression in HCT116 cells overexpressing *YY2* and/or *YY1*, as determined using qRT-PCR. β-actin was used for qRT-PCR normalization control and as western blotting loading control. **(C)** Schematic diagram of DNA affinity precipitation assay using biotin-DNA probe targeting the -672 to -662 region of the *SLC7A11* promoter. Quantification data are shown as mean ± SD (n = 3). *P* values were calculated using one-way ANOVA analyses. pcCon: pcDNA3.1(+); ***P* < 0.01.

Figure S9.



Supplementary Fig. S9. YY2 regulates SLC7A11/GSH synthesis axis in *YY1*-knocked down cells. (A) YY1 mRNA (left pannel) and protein (right pannel) expression levels in *YY1*-knocked down HCT116 stable cell lines, as determined using qRT-PCR and western blotting, respectively. **(B)** Viabilities of *YY1*-knocked down HCT116 cells overexpressing *YY2* were measured at indicated time points. **(C)** SLC7A11 mRNA (left) and protein (right) expression levels in *YY1*-knocked down HCT116 cells overexpressing *YY2*, as determined using qRT-PCR and western blotting, respectively. **(D)** Cysteine (left) and GSH (right) levels in *YY1*-knocked down HCT116 cells overexpressing *YY2*. **(E)** Lipid peroxidation in *YY1*-knocked down HCT116 cells overexpressing *YY2*, as assessed by C11-BODIPY staining and flow cytometry. Cells transfected with shCon or pcCon were used as controls. β-actin was used for qRT-PCR normalization and as western blotting loading control. Quantification data are shown as mean ± SD (n = 3). *P* values were calculated using two-tailed unpaired Student's t-test. pcCon: pcDNA3.1(+); $^{**}P < 0.01$.

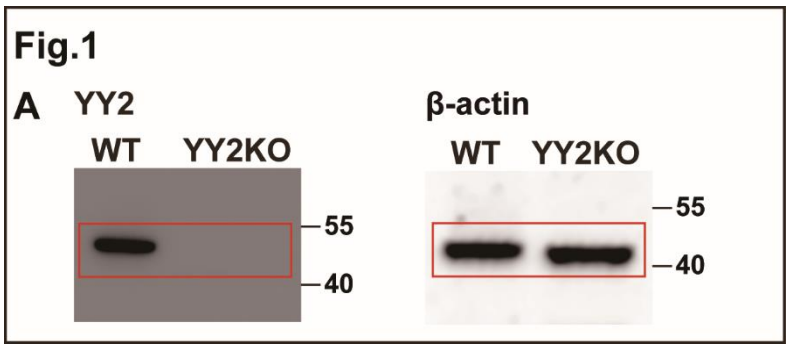
Figure S10.



Supplementary Fig. S10. YY1 regulates ferroptosis in HCT116^{YY2null} cells. (**A**) Cysteine (left) and GSH (right) levels in HCT116^{YY2null} cells overexpressing *YY1*. (**B**) Lipid peroxidation in HCT116^{YY2null} cells overexpressing *YY1*, as assessed by C11-BODIPY staining and flow cytometry. **(C)** Viabilities of HCT116^{YY2null} cells overexpressing *YY1* were measured at indicated time points. Cells transfected with pcCon were used as controls. Quantification data are shown as mean ± SD (n = 3). *P* values were calculated using two-tailed unpaired Student's t-test. pcCon: pcDNA3.1(+); YY2KO: HCT116^{YY2null} cells; $^{**}P < 0.01$.

Figure S11.

A



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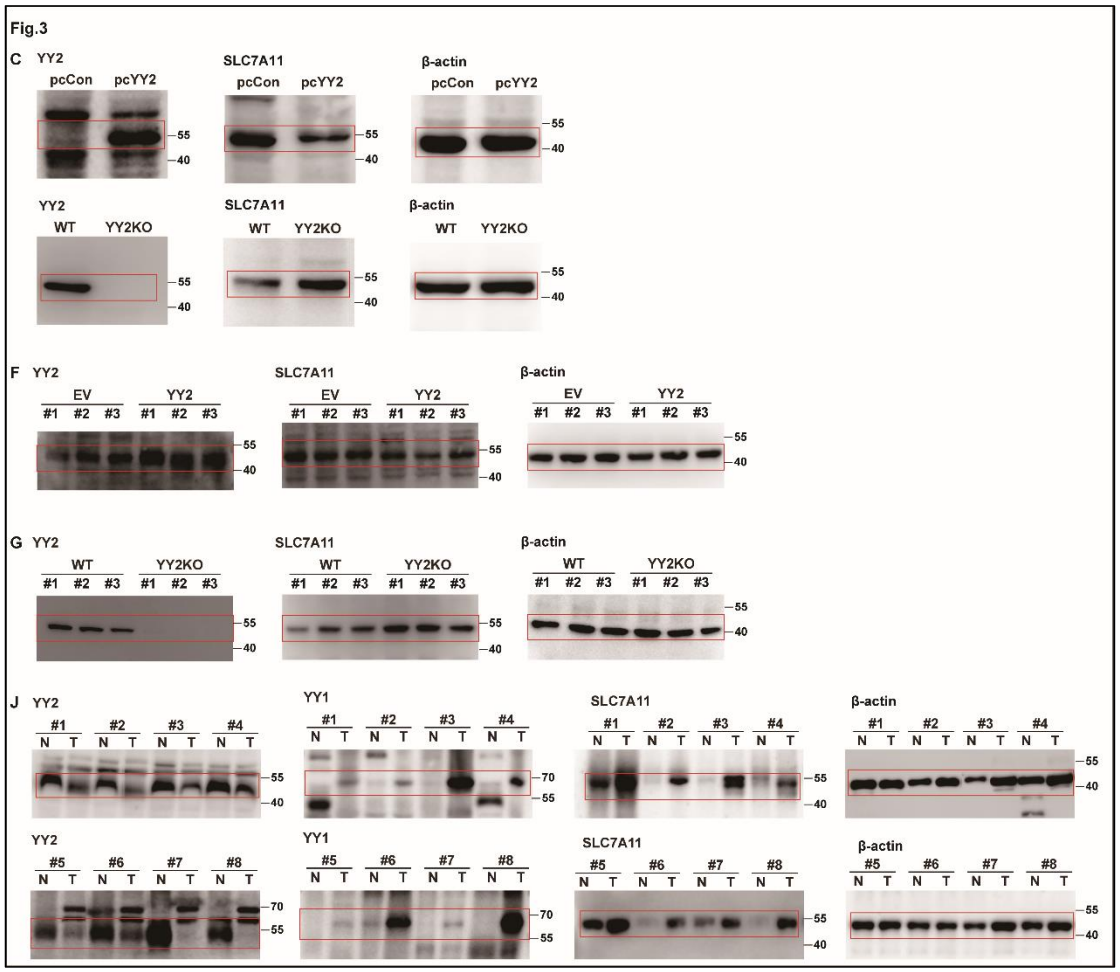
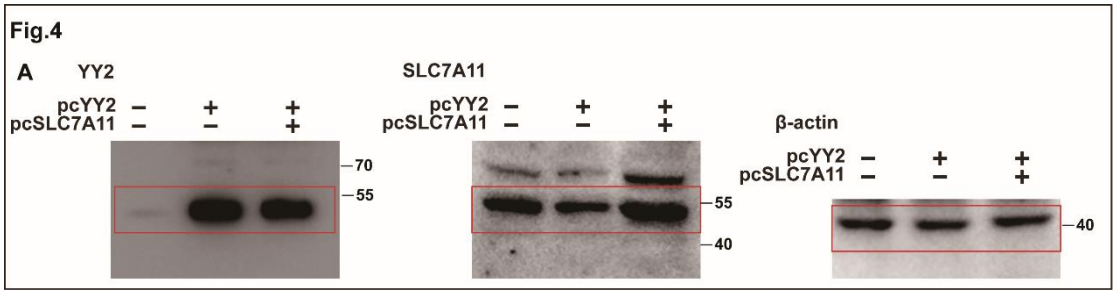


Supplementary Fig. S11. Uncropped western blots with the indicated areas of selection in Figures 1, 3 to 6 and 8, Figures S1, S2, and S4 to S9. (continued)

C



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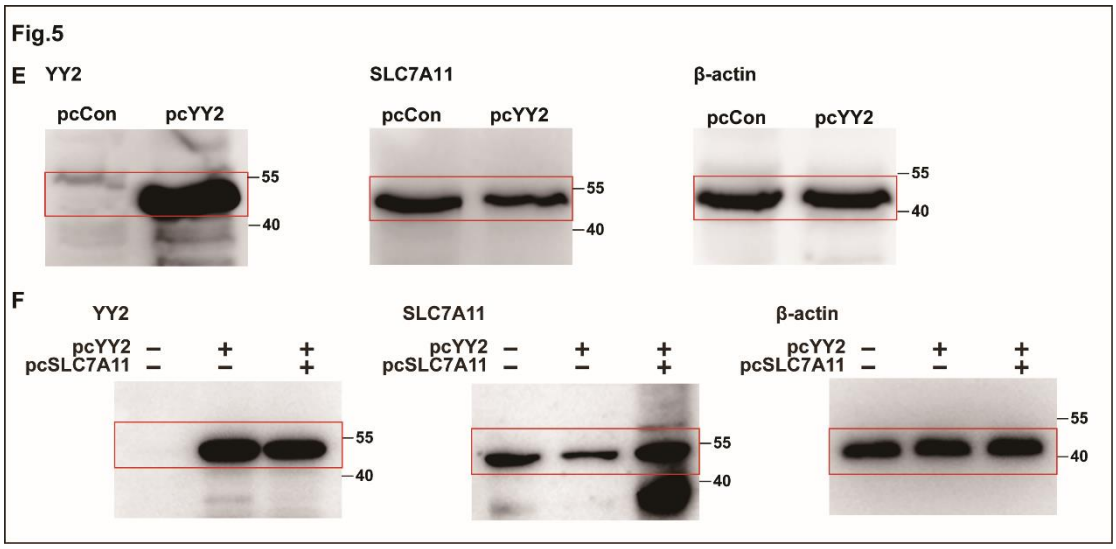

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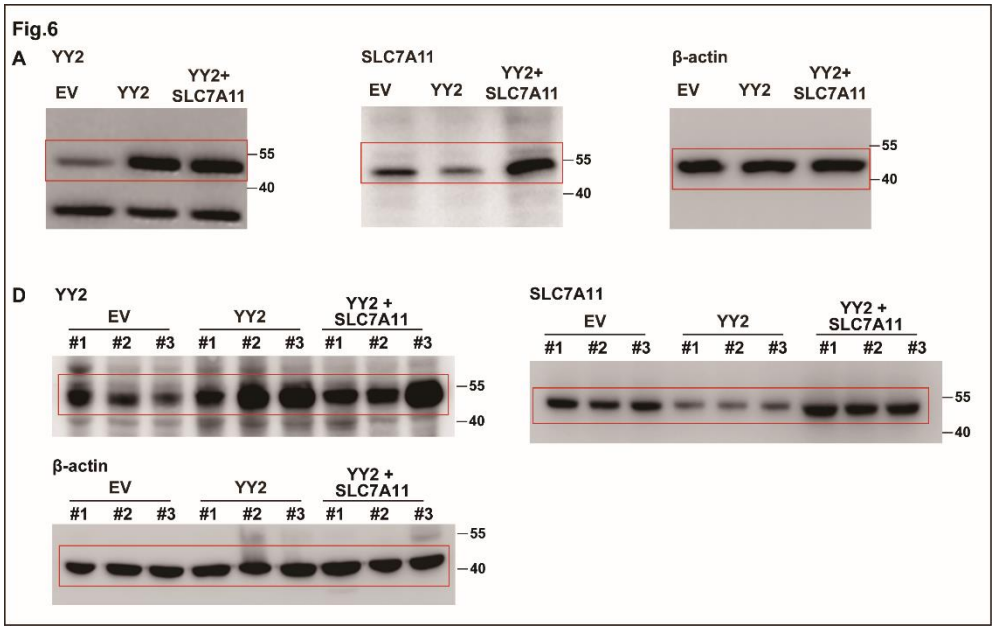
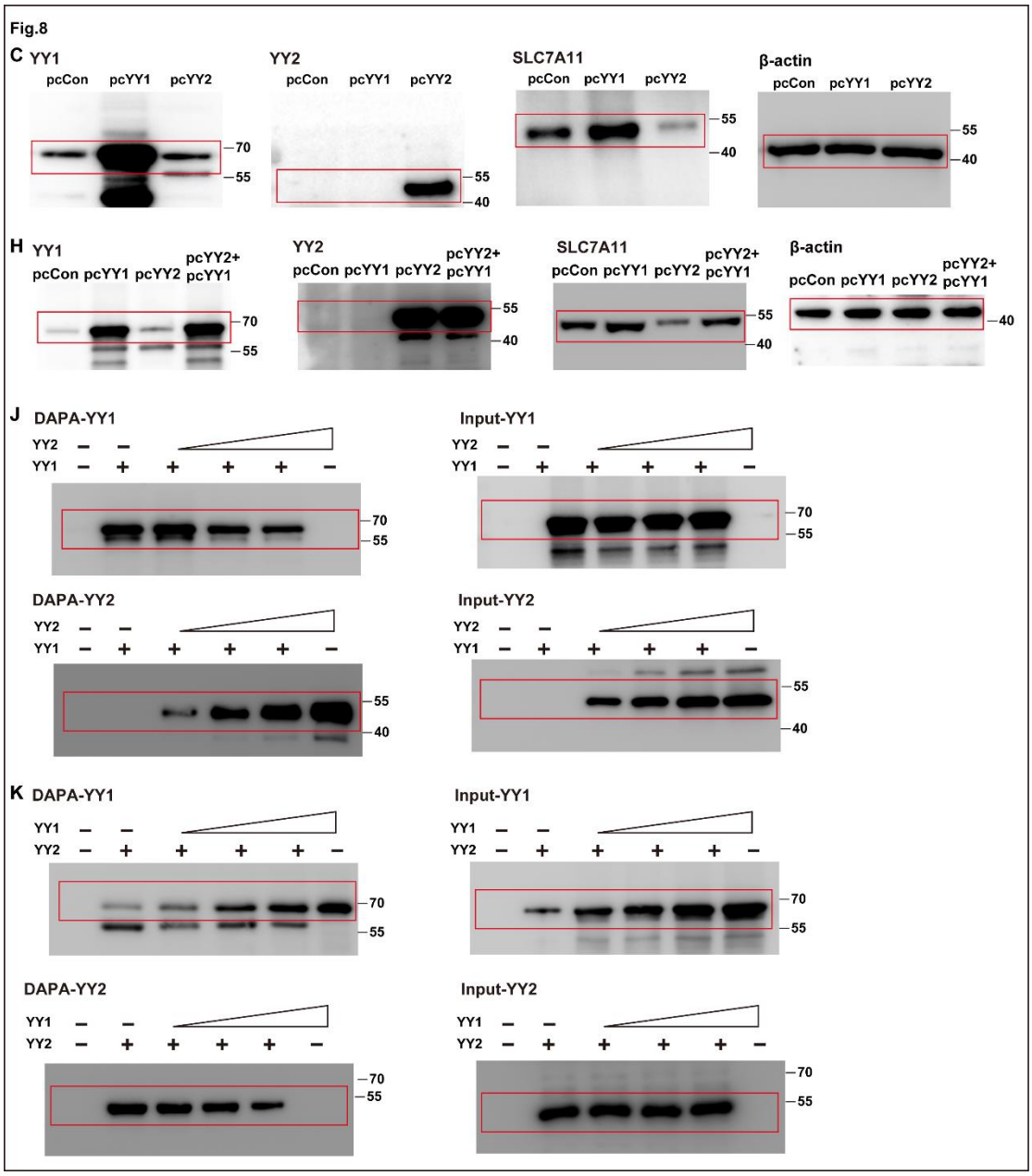


Supplementary Fig. S11. Uncropped western blots with the indicated areas of selection in Figures 1, 3 to 6 and 8, Figures S1, S2, and S4 to S9. (continued)

F



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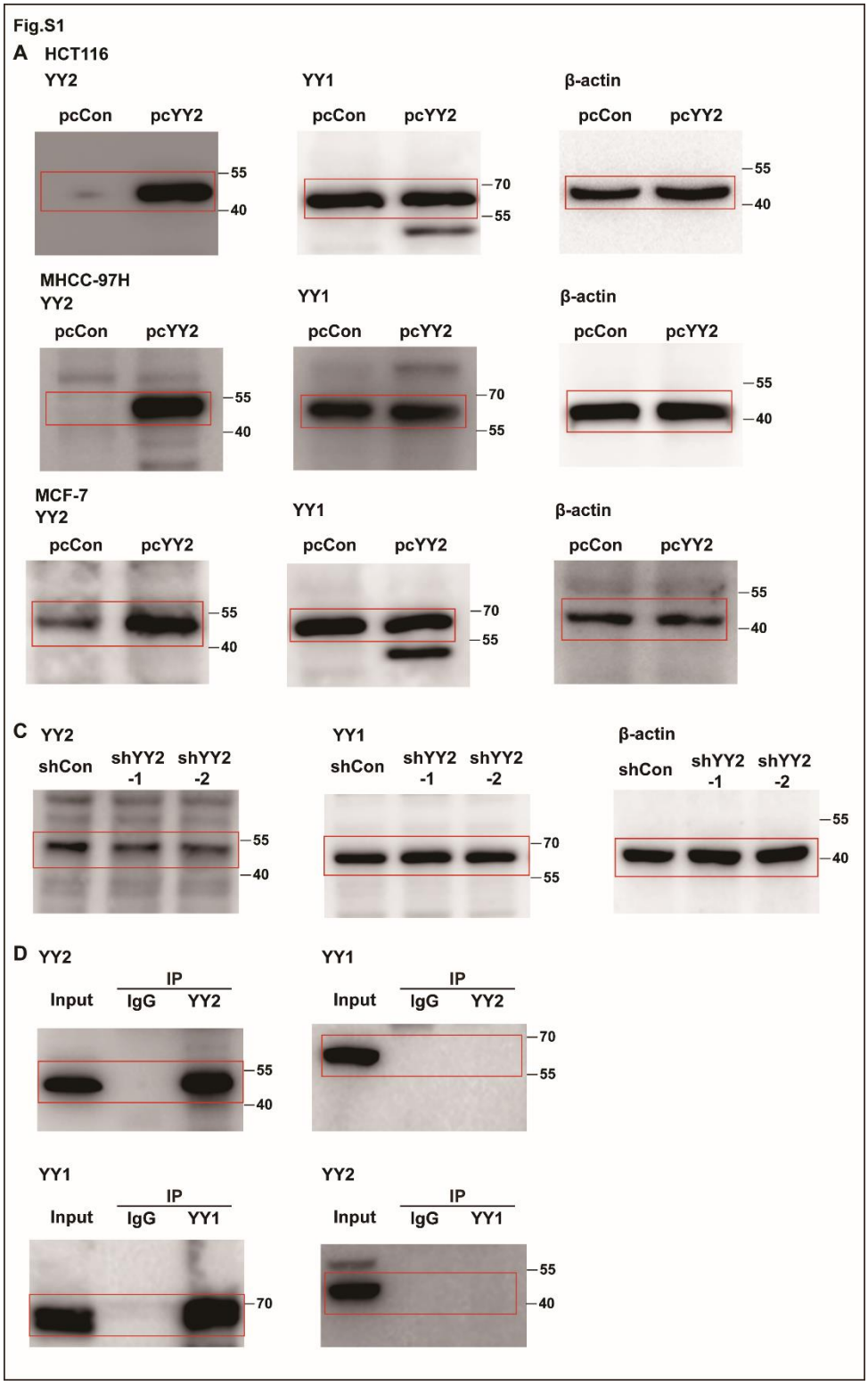

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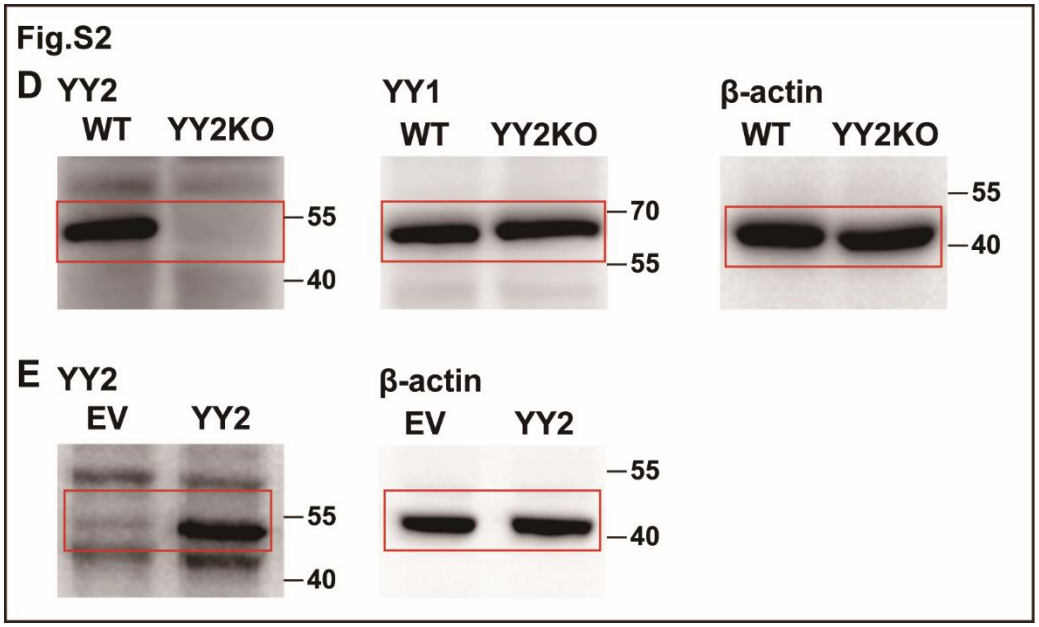

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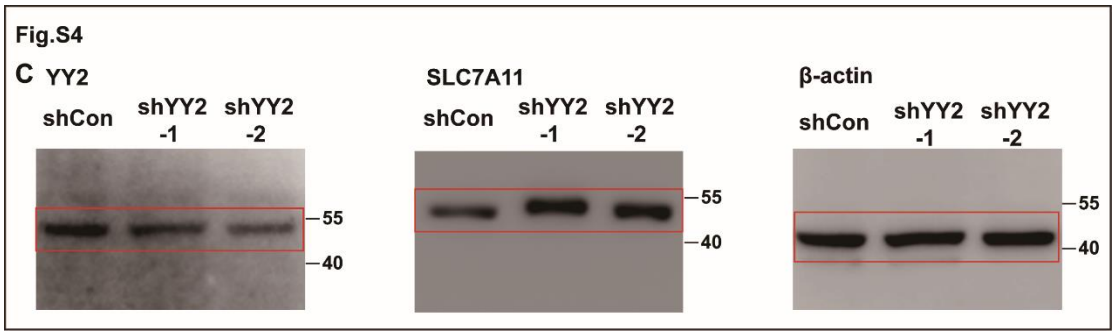
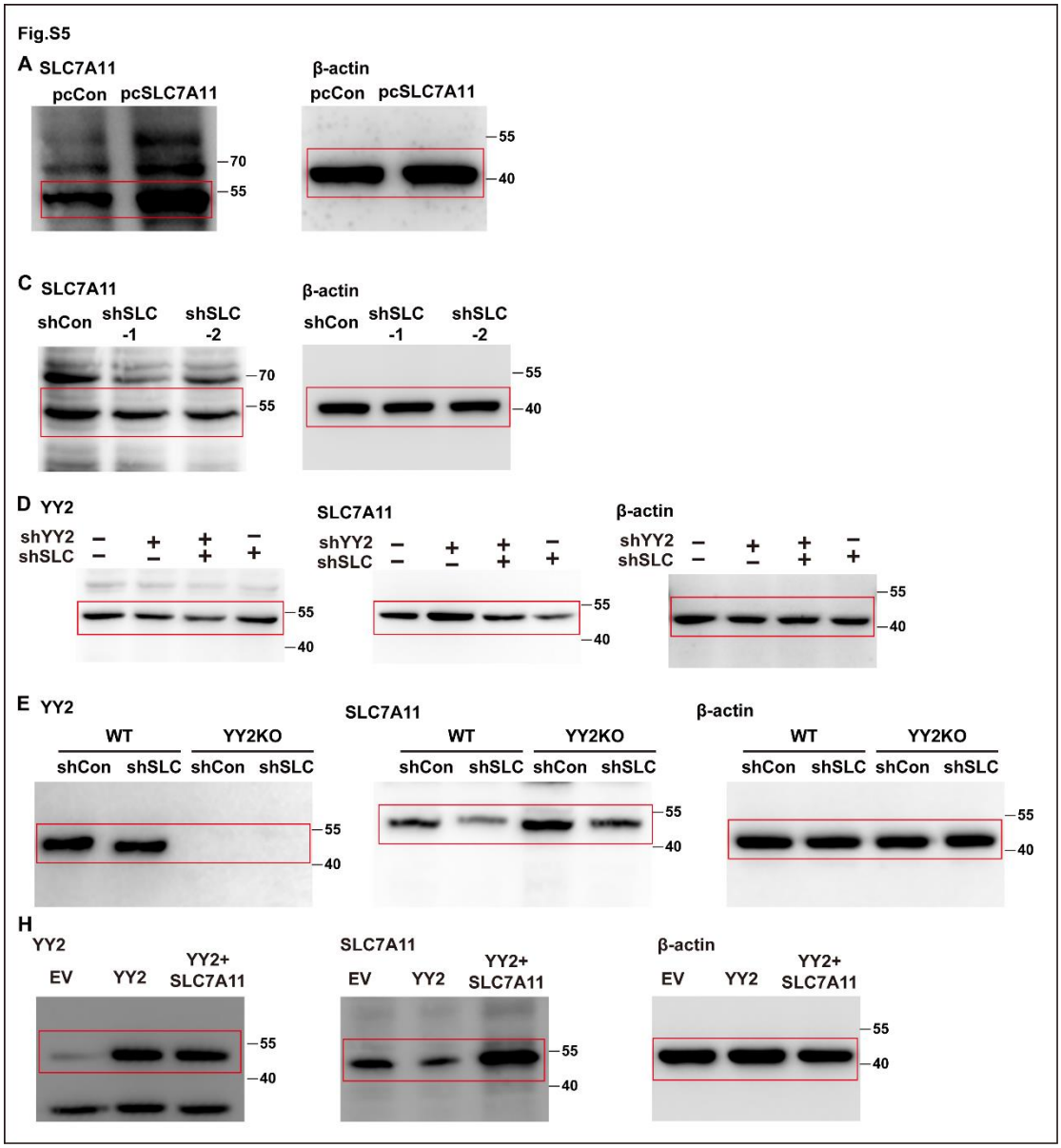


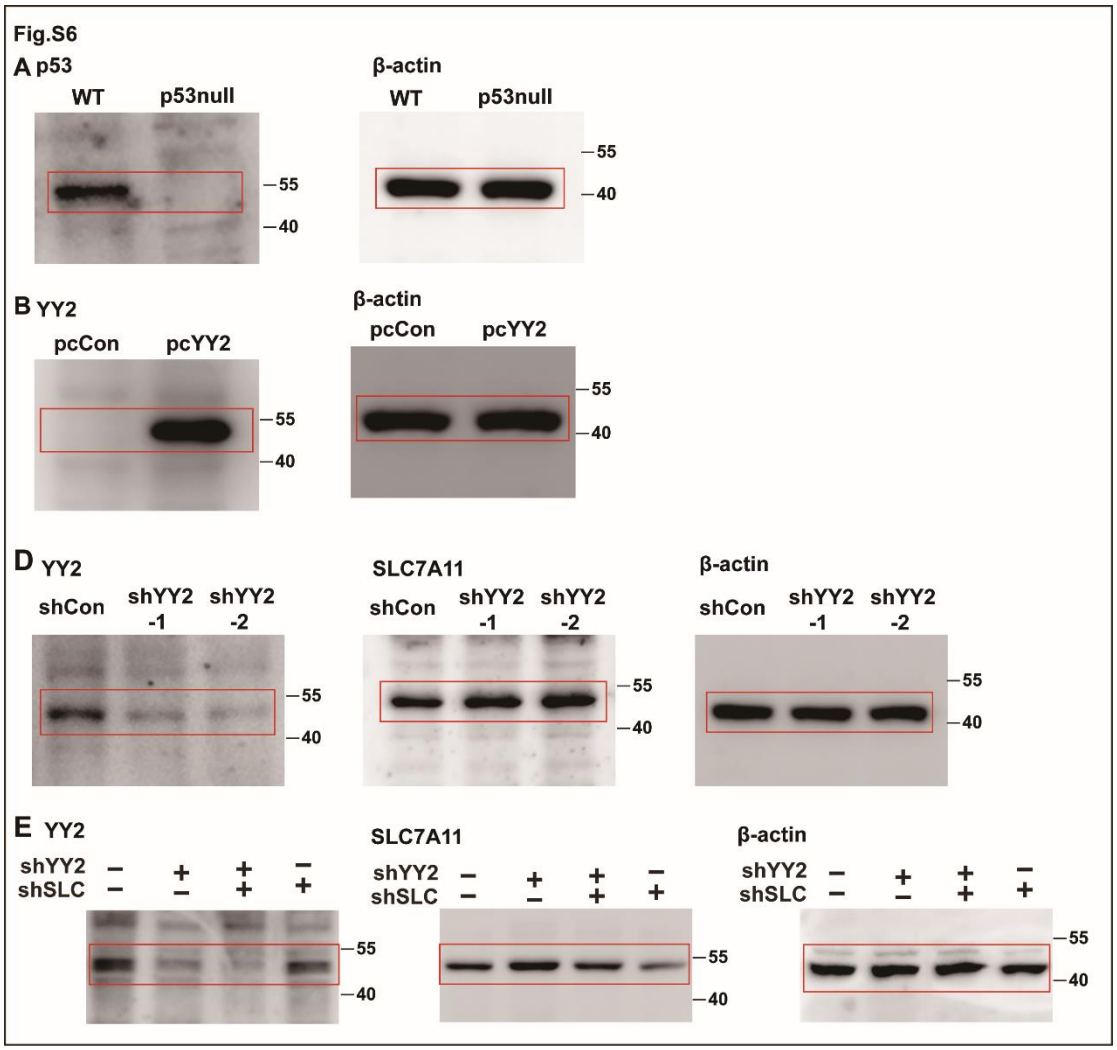
Supplementary Fig. S11. Uncropped western blots with the indicated areas of selection in Figures 1, 3 to 6 and 8, Figures S1, S2, and S4 to S9. (continued)

J



Supplementary Fig. S11. Uncropped western blots with the indicated areas of selection in Figures 1, 3 to 6 and 8, Figures S1, S2, and S4 to S9. (continued)

K



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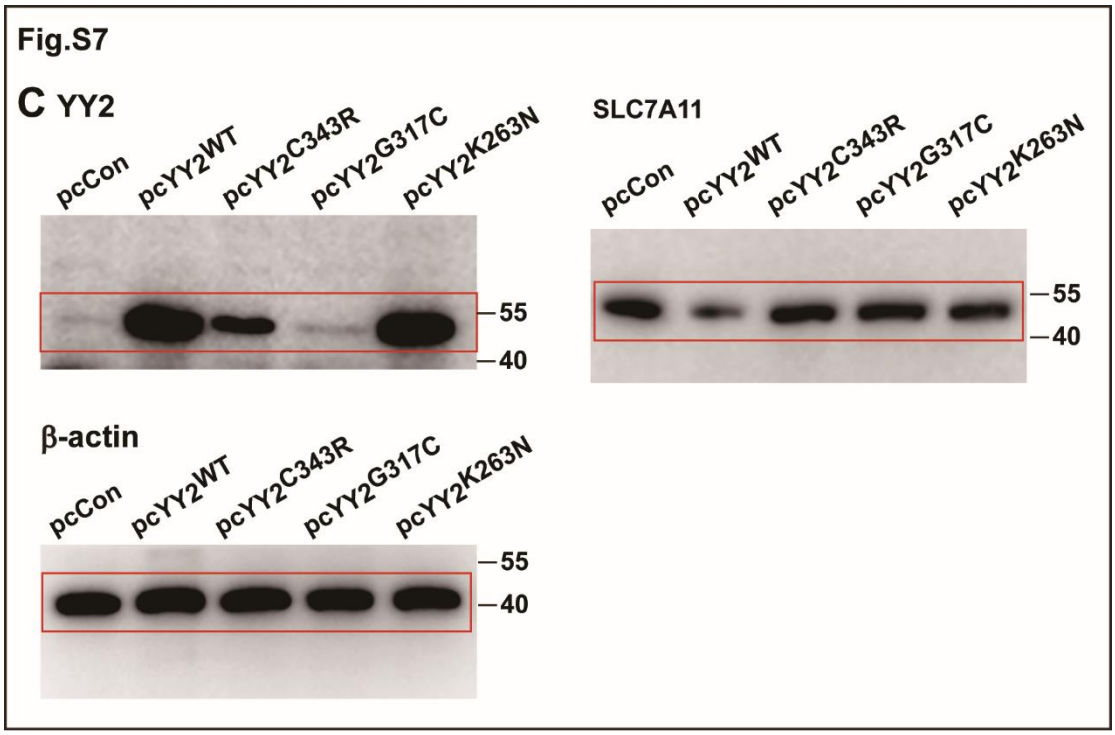

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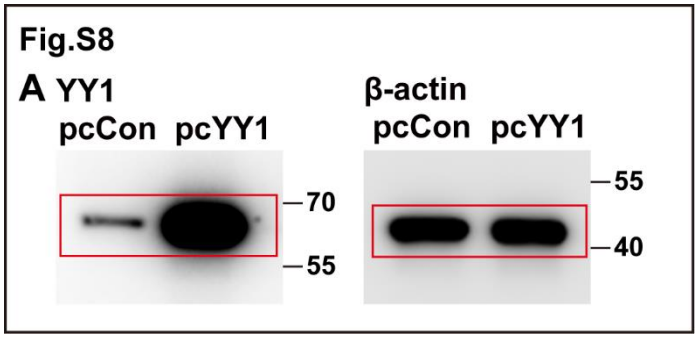
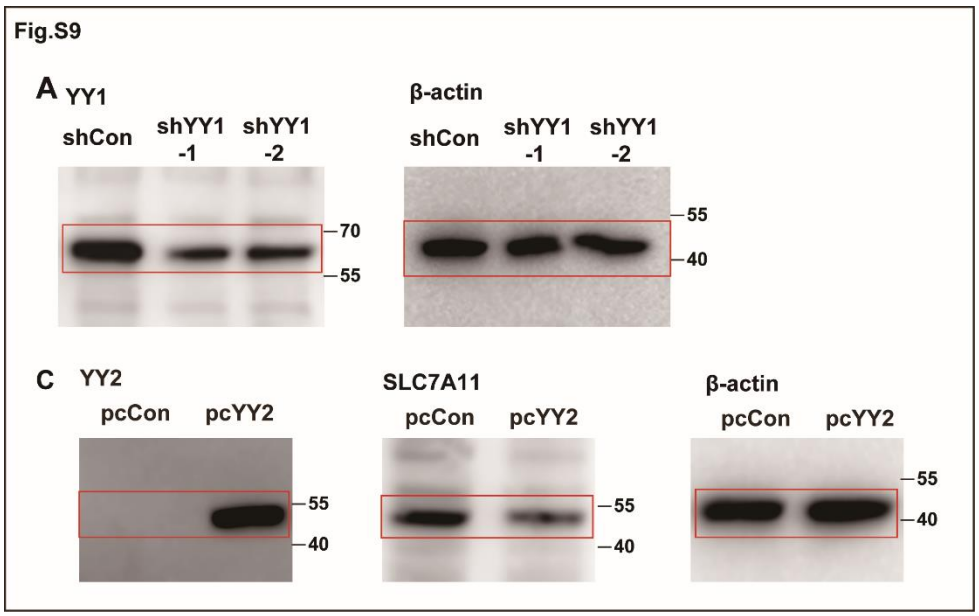


Supplementary Fig. S11. Uncropped western blots with the indicated areas of selection in Figures 1, 3 to 6 and 8, Figures S1, S2, and S4 to S9. (continued)

N



Supplementary Fig. S11. Uncropped western blots with the indicated areas of selection in Figures 1, 3 to 6 and 8, Figures S1, S2, and S4 to S9. (continued)

Table S1. Primer pairs used for gene quantification by quantitative RT-PCR.

Genes	Refseq No.	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
YY2	NM_206923.4	GAAGTGGTGGGCTATTGCGA	AGGGTCATCTGGAAGTGCTC
YY1	NM_003403.4	GCGGAGCCCTCAGCCATGGCCTCG	CAGCGGCTGCAGAGCGATCATGG
SLC7A11	NM_014331.4	TCCTGCTTTGGCTCCATGAACG	AGAGGAGTGTGCTTGCGGACAT
SLC40A1	NM_014585.6	CAGTTTGCAACATGTCTGTACC	TAGCAACGTATTGCAGTCTCC
SLC39A8	NM_001135147.1	CTGCAATACCAGCACCTTCA	CACACACAGCATCACGTCTC
SAT1	NM_213613.4	GTCTTTGCCTAACGTCGCTG	TTAGGGAGGCGGTGCTTAGA
FTL	NM_000146.4	CAACCAACCATGAGCTCCCA	TTTCATGGCGTCTGGGGTTT
SLC3A2	NM_002394.6	GTTTGTCTCAGGCAAGGCTC	GGAACAAGGAAAGGAGGGAG
GCLC	NM_001498.4	GAGGTCAAACCCAACCCAGT	AAGGTACTGAAGCGAGGGTG
ACSL3	NM_004457.5	TGACACAAGGGCGCATATCT	CCAGTCCTTCCCAACAACGA
LCPAT3	NM_005768.6	TGTGGAAAGACAGGCTGCCA	GAAGAGGCAGAAGGCAGTCAT
β-actin	NM_001101.3	CGAGCGCGGCTACAGCTT	TCCTTAATGTCACGCACGATTT

Table S2. Antibodies used for western blotting, immunohistochemical staining and ChIP assay.

Antibody	Product number	Maker	Experiment	Dilution
Anti-YY2	sc-374455	Santa Cruz Biotechnology	Western blotting Immunohistochemistry Chromatin Immunoprecipitation Immunoprecipitation	1/1000 1/100 30 μg/mL cell lysate 25 μg/mL cell lysate
Anti-YY1	22156-1-AP	Proteintech	Western blotting Immunohistochemistry Chromatin Immunoprecipitation Immunoprecipitation	1/1000 1/200 30 μg/mL cell lysate 25 μg/mL cell lysate
Anti-SLC7A11	26864-1-AP	Proteintech	Western blotting Immunohistochemistry	1/1500 1/500
Anti-p53	60283-2- Ig	Proteintech	Western blotting	1/1000
Anti-β-actin	60008-1- Ig	Proteintech	Western blotting	1/100000
Anti-histone H3	17168-1-AP	Proteintech	Chromatin Immunoprecipitation	30 μg/mL cell lysate
Anti-4-HNE	CABT-B1068	Creative Diagnostics	Immunohistochemistry	1/500
Goat anti-rabbit IgG	ZB2301	ZSGB-BIO	Western blotting	1/10000
Goat anti-mouse IgG	ZB2305	ZSGB-BIO	Western blotting	1/10000