SUPPLEMENTAL INFORMATION

LSD1 destabilizes FBXW7 and abrogates FBXW7 functions independent of its demethylase activity

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MATERIALS AND METHODS

Cell culture

H1299, A549, A427 and H358 lung cancer cell lines, and HEK293 cell line were purchased from American Type Culture Collection. Isogenic HCT116 cells with or without FBXW7 (HCT116-*FBXW7*$^{+/+}$ and HCT116-*FBXW7*$^{-/-}$) were gift from Dr. B. Vogelstein. A427 and H358 were grown in RPMI-1640 with 10% FBS. H1299, A549, HEK293 and DLD1 were cultured in DMEM with 10% FBS, and HCT116 cells were cultured in McCoy's 5A with 10% FBS. All cell lines were tested to be free of mycoplasma contamination.

ATPlite proliferation assay

Cells were seeded into 96-well plates with 3,000 cells per well in triplicate and grown for 72h, followed by ATPlite proliferation assay (Perkin-Elmer), according to the manufacturer's instructions.

Colony formation assay

Cells were seeded into 60-mm dishes with 800 cells per dish in triplicate, and cultured at 37 ℃ for 14 d. The colonies were fixed with 10% acidic acid in methanol, stained with 0.05% methylene blue, and counted.

Antibodies and Immunoblotting

Immunoblotting analysis were performed as described (1). The antibodies used were as follows: LSD1 (Cell signaling), FBXW7 (Bethyl), FLAG (Sigma), HA (Roche), His (Cell signaling), GST (Cell signaling), c-Myc (Santa Cruz), NOTCH-1 (Cell signaling), MCL-1 (Cell signaling), XRCC4 (BD Transduction Laboratories), c-JUN

(Santa Cruz), GFP (Cell signaling), p-ERK (Cell signaling), p62 (Cell signaling), HSC-70 (Cell signaling), β-actin (Santa Cruz).

Immunoprecipitation

Whole cell extracts were incubated with indicated antibody for 3 hrs followed by incubation with protein A&G beads (Santa Cruz) at 4°C overnight with rotation. The beads were washed three times with lysis buffer, and the immunoprecipitation complexes were subjected to SDS-PAGE.

RNA preparation and SYBR green quantitative real-time PCR

Total RNA was isolated using TRIzol® Reagent (Ambion, 15596-026, L ife-Technologies, USA). Quantitative real-time PCR analysis was carried out as described (1) with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control of RNA integrity. The primer for FBXW7 is: 5'-AGAG GAGGAACAGCAACAGC-3' (forward); 5'-TGGGGAGGAGAGTTGGTGAA-3' (reverse).

siRNA transfection

The sequences for siLSD1-1# is: GCCTAGACATTAAACTGAATA; siLSD1-2# is: GCTACATCTTACCTTAGTCAT; FBXW7-1# is: TGATACATCAATCCGTG TTTG; siFBXW7-2# is: CCTAAAGAGTTGGCACTCTAT. siRNA pool for p62/SQSTM1 was purchased from Santa Cruz (catalog no. sc-29679) and control siRNA (catalog no. sc-37007). Cells were transfected with siRNA duplexes (10 nM) using Lipo2000 transfection reagent (Life Technologies) according to the manufacturer's instructions.

LEGENDS FOR SUPPLEMENTAL FIGURES

Figure S1. FBXW7 binds to LSD1, but fails to promote its ubiquitylation

(**A&B**) The FBXW7-LSD1 binding: A549 cells were subjected to IP with LSD1-Ab (A), FBXW7-Ab (B) or control IgG, followed by IB with indicated Abs.

(**C**) FBXW7 binds to LSD1 via the WD40 domain. Schematic representation of FLAG-FBXW7 domain structures (Top). Two FLAG-fused domain constructs of FBXW7 were transfected with or without HA-LSD1 into 293 cells for 48 hrs. Cells were subjected to IP with FLAG-Ab (Mouse), followed by IB with HA-Ab and FLAG-Ab (Rabbit) (Bottom).

(**D**) LSD1 binds to FBXW7 via the C-terminus. Schematic representation of FLAG-LSD1 domain structures (Top). Three FLAG-fused domain constructs of LSD1 were transfected with or without HA-FBXW7 into 293 cells for 48 hrs. Cells were subjected to IP with FLAG-Ab (Mouse), followed by IB with HA-Ab and FLAG-Ab (Rabbit) (Bottom).

(**E&F**) FBXW7 overexpression has no effect on LSD1 levels. A549 cells were transfected with increasing amounts of HA-FBXW7 (E), or fixed amount of HA-FBXW7, followed by CHX treatment with indicated periods of time (F), then IB with indicated Abs.

(**G**) FBXW7 deletion has no effect on LSD1 half-life. Paired HCT-116 cells with *WT* or *FBXW7-null* were treated with CHX for indicated time periods and harvested for IB.

(**H**) Genetic Fbxw7 depletion has no effect on the protein levels of Lsd1. Two pairs of

MEF cells derived from little-mate embryos of Fbxw7-fl/fl mice were either infected with Ad-GFP control (+/+) or Ad-Cre to delete floxed Fbxw7 allele (-/-), followed by IB with indicated Abs.

(**I**) FBXW7 has no effect on demethylase activity of LSD1. The *in vitro* demethylase assay was performed by incubating peptides (H3K4-Me2, AA1-21) with purified FLAG-LSD1 in the presence of absence of purified HA-FBXW7. The reaction mixture was subject to Dot blotting or IB with indicated Abs. SE: Short exposure; LE: Long exposure.

Figure S2. LSD1 destabilizes FBXW7 via both proteasome and lysosome pathways

(**A&B**) Overexpression of LSD1 decreases the protein levels of FBXW7. A549 cells (A) or paired HCT116 cells (B) were transfected with increasing amounts of FLAG-LSD1 and HA-FBXW7, followed by IB with indicated Abs.

(**C**) LSD1 inhibits FBXW7 E3 ligase activity. The 293 cells were transfected with indicated plasmids, lysed under denatured condition at 6M guanidinium solution, followed by Ni-beads pull-down and IB to detect polyubiquitylation of c-JUN.

(**D**) LSD1 inhibits FBXW7 binding with its substrates. H1299 cells were transfected with or without FLAG-LSD1 for 24h, followed by treatment with MG132 and CQ for 12h, then subjected to IP with FBXW7-Ab or control IgG, followed by IB with indicated Abs.

(E) LSD1 fails to bind with cyclin E. H1299 cells were transfected with FLAG-LSD1 for 48h, then subjected to IP with FLAG-Ab or control IgG, followed by IB with

indicated Abs.

(F) LSD1 reduction of FBXW7 is independent of its demethylase activity. H1299 cells were co-transfected with FLAG-LSD1 and HA-FBXW7 for 48h, then treated with MG132 or different LSD1 inhibitors (Compound 6b: 1 μM for 24h; GSK2879552: 0.3 μM for 24h), followed by IB with indicated Abs.

(**G**) LSD1 has no effect on FBXW7 mRNA. A549 cells were transfected with increasing amounts of FLAG-LSD1 (1 μg and 3 μg) for 48 hours, then harvest cells for total RNA isolation and qRT-PCR analysis. Shown is mean ± SEM from three independent experiments.

(**H**) LSD1 reduction of FBXW7 is independent of its demethylase activity. H1299 cells were co-transfected with FLAG-FBXW7 and HA-LSD1-K661A (a demethylase dead mutant), treated with MG132 or CQ in the last 6 h before harvesting, followed by IB.

(**I&J**) LSD1 shortens FBXW7 half-life, rescued by MG132 and CQ: Cells were co-transfected FBXW7 with LSD1 or vector control as indicated, then treated with CHX for indicated periods (I) in the absence or presence of MG132 and/or CQ (J), followed by IB. SE: Short exposure; LE: Long exposure.

Figure S3. LSD1 promotes FBXW7 self-ubiquitylation by disrupting its dimerization

(**A**) Pure E1, E2 (UBCH5C and UBE2M), E3 and substrate (FBXW7-WT or ΔF) were incubated with or without FLAG-LSD1 (WT or K661A) in a reaction mixture containing ATP and ubiquitin for 1 hr, followed by IB with HA-Ab.

(B) T205A mutation does not affect the FBXW7-LSD1 binding. H1299 cells were co-transfected with FLAG-LSD1 and HA-FBXW7-WT or HA-FBXW7-T205A mutant, followed by IP with FLAG-Ab or control IgG, and IB with indicated Abs.

(**C**) FBXW7 D-domain deletion mutant is unstable. Cells were transfected with GFP-FBXW7-WT or ΔD mutant, then switched 48 later to fresh medium containing CHX for indicated periods and harvested for IB.

(**D**) FBXW7 D-domain deletion mutant has reduced E3 activity toward substrate, but increase E3 activity toward self-ubiquitylation. The 293 cells were transfected with indicated plasmids, harvested 15 min post-IR and lysed under denatured condition at 6M guanidinium solution, followed by Ni-beads pull-down and IB to detect XRCC4 polyubiquitylation or self-ubiquitylation.

(**E**) LSD1 knockdown increases FBXW7 dimerization. The 293 cells were transfected with si-LSD1 and FBXW7 constructs with different tags as indicated, followed by IP with FLAG-Ab and IB with the indicated Abs.

(**F**) LSD1 disrupts dimerization of FBXW7, but not β-TrCP. H1299 cells were transfected with indicated plasmids, followed by MG132+CQ treatment. Cells were subjected to IP with HA-Ab, and IB with indicated Abs.

(**G**) LSD1 disrupts FBXW7 dimerization *in vitro*. The 293 cells were co-transfected with indicated plasmids, then treated with nocodazole (50ng/ml) 48h post-transfection for 8h. The anti-FLAG/HA immunoprecipitation was performed to affinity-purify LSD1/FBXW7 proteins. Eluted HA-FBXW7 proteins were incubated with increasing amounts (0, 0.3, 1, or 3 μM) of eluted FLAG-LSD1 (WT/M2) proteins before GFP-

FBXW7 IP was performed. The resolving HA-FBXW7/GFP-FBXW7 dimer was analyzed by IB.

Figure S4. Ubiquitylated FBXW7 is degraded by both proteasome and p62-mediated lysosome pathways

(**A&B**) Autophagy induction reduces FBXW7 levels. H1299 cells were pretreated with MG132 for 2h, then switched to fresh medium without FBS (A) or treated with rapamycin (B) for indicated periods, and harvested for IB.

(**C**) p62 does not bind to non-ubiquitylated FBXW7. The 293 cells were transfected with indicated plasmids for 48h, lysates were subjected to IP with HA-Ab and followed by IB.

Figure S5. LSD1 inhibits the cellular functions of FBXW7

(**A-D**) H1299 or A549 cells were transfected with indicated plasmids or siRNAs for 48h, then plated into 96-plates in triplicates, and cultured for 72h, followed by ATPlite proliferation assay. Shown are mean ± SEM, n=3, *p<0.05, ** p<0.01; *** p<0.001.

(**E&F**) H1299 or A427 cells were transfected with FLAG-LSD1-WT or its indicated mutants, followed by clonogenic assay (n=3). *p<0.05. NS, no significance.

(**G-I**) H1299, A549 or A427 cells were transfected with indicated plasmids for 48h, then switched to fresh medium containing 10% FBS for 72h, followed by ATPlite proliferation assay. *p<0.05, ** p<0.01; *** p<0.001. NS, no significance.

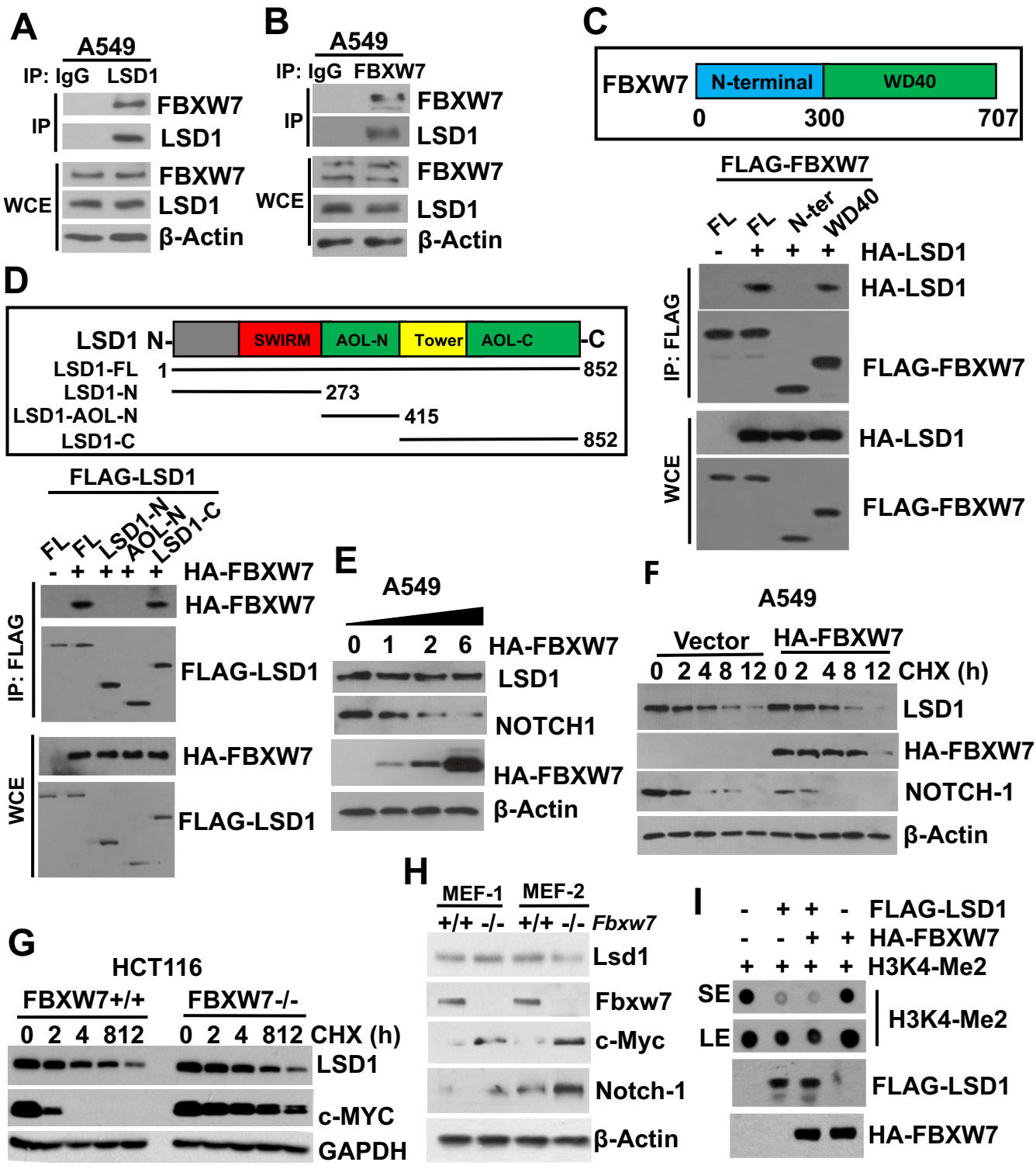
(**J**) LSD1 inhibits FBXW7-induced NHEJ repair. Cells were transfected with indicated plasmids for 48h, then transfected with linearized pEYFP plasmid (by NheI digestion),

and analyzed by qPCR 12h later using one set of primers (designated as P2), flanking the ligated pEYFP region. Shown is mean ±SEM from three independent experiment with normalization to an uncut flanking DNA sequence. *p<0.05, ** p<0.01; *** p<0.001. NS, no significance.

References

1. Lan H, Tang Z, Jin H, & Sun Y (2016) Neddylation inhibitor MLN4924 suppresses growth and migration of human gastric cancer cells. *Sci Rep* 6:24218.

Lan et al, Figure S1



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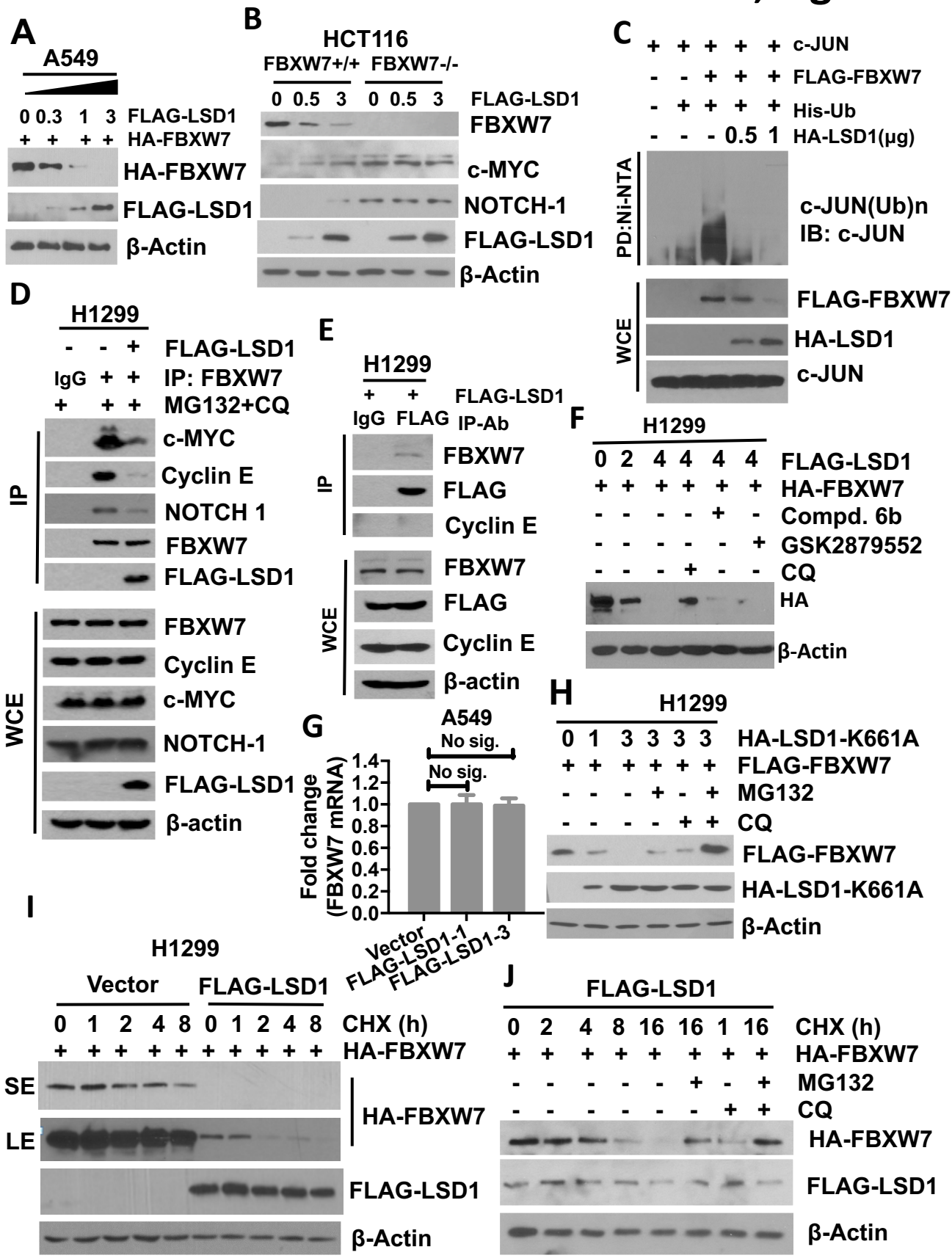

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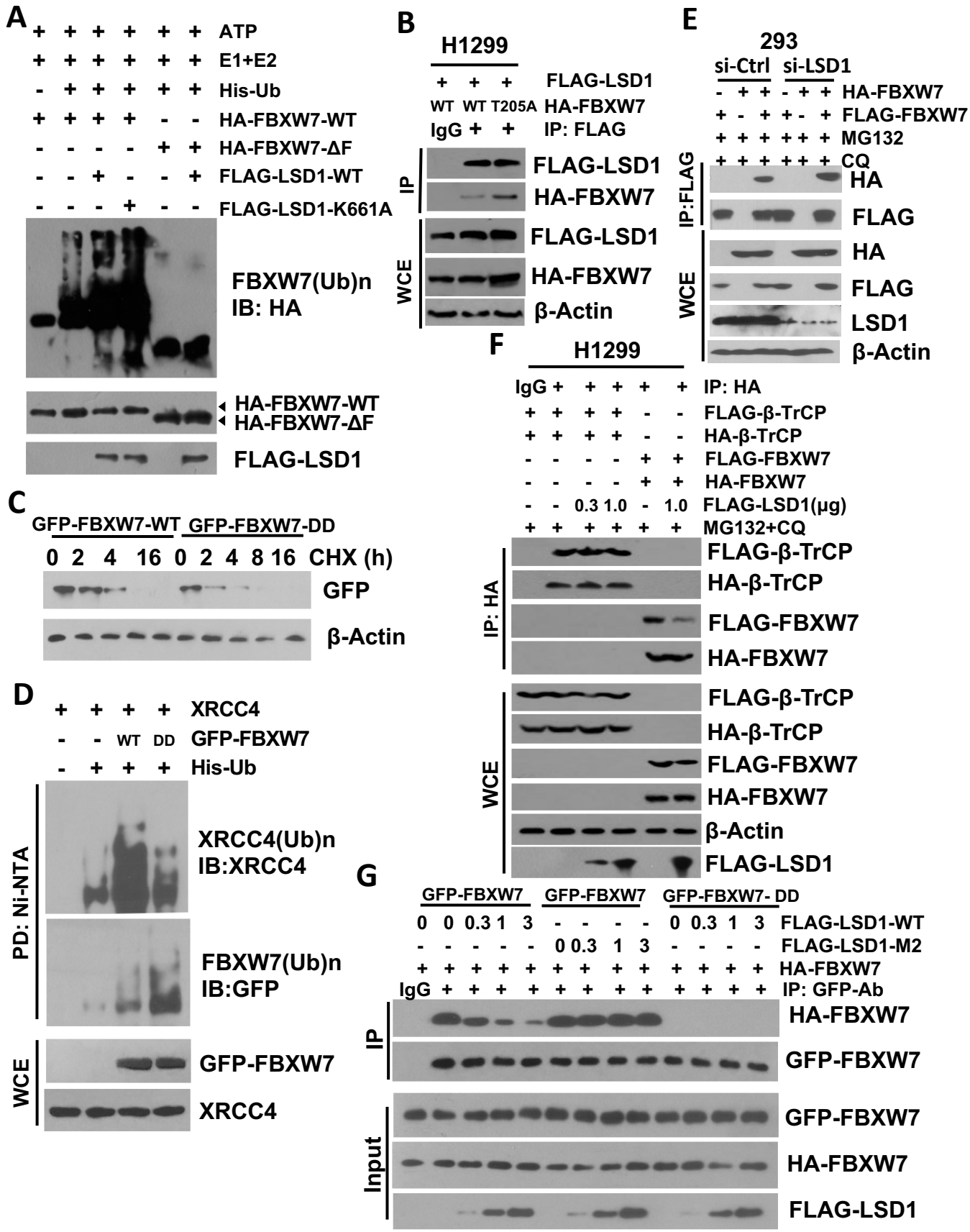

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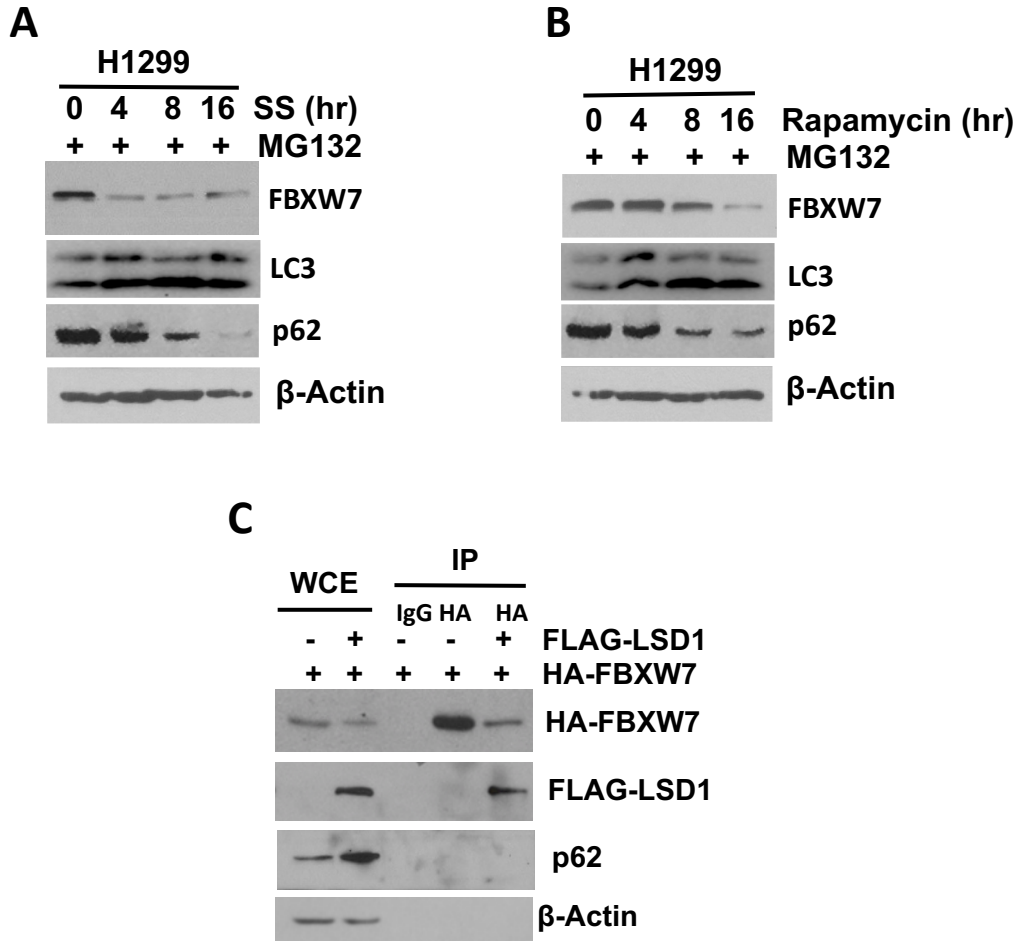

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