COP9 signalosome subunit 6 promotes tumorigenesis through stabilization of MDM2

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SUPPLEMENTAL MATERIALS AND METHODS

Genome Structure Analyses

For array-CGH analysis, SIGMA (System for Integrative Genomic Microarray Analysis, http://sigma.bccrc.ca) was performed to examine the gain and loss of *CSN6* genomic loci (42). Results were represented by BCCRC SMRT arrays in 17 breast cancer cell lines and 158 multiple type cancer cell lines by genome karyogram using the March 2006 genomic build.

Plasmids, Antibodies and Reagents

The PCR-generated DNA fragments of human *p53*, and *MDM2* genes were sub-cloned into pCMV5 to yield constructs that encode indicated proteins. The PCR-generated DNA fragments of human *CSN6* gene was sub-cloned into pCMV5 to generate a construct that encodes CSN6 with a Flag-tagged sequence or into pCDNA6 with a Myc-tagged sequence.

The antibodies were used in the experiments are: CSN6 (BIOMOL international, PW8295), MDM2 [Santa Cruz Biotechnology, SMP-14 (sc-965), N-20 (sc-813); CALBIOCHEM, OP115T], p53 [Santa Cruz Biotechnology, DO-1 (sc-126), FL-393 (sc-6243); CALBIOCHEM, PC35], Flag (Sigma, F3165), Myc (Roche, 11667203001), HA (Roche, 11666606001), p21 (Santa Cruz Biotechnology, F-5 sc-6246), His (Cell Signaling Technology, 2365), Ubiquitin (Zymed, 13-1600), PARP (Cell Signaling Technology, 9542), PUMA (ProSci Incorporated, 3041), CSN5 (GeneTex, MS-JAB11-PX2), GFP (Santa Cruz Biotechnology, sc-9996), α-tubulin (Sigma, T-5168), and actin (Sigma, A2066). Cycloheximide (C4895), MG132 (C2211), Puromycin (P7255) and Doxorubicin (D1515) were purchased from Sigma. Blasticidin (ant-bl-1) was purchased from InvivoGen.

Quantitative-PCR

Primers for Q-PCR of genomic loci of *CSN6* (5'-CACCTGACCCCTCGGACAT; 5'-AAAGAGGGGGCTCTCGATGA) and *MDM2* (5'-CCTTCGTGAGAATTGGCTTC; 5'-CAACACATGACTCTCTGGAATCA) were as referred by Primer Bank (http://pga.mgh.harvard.edu/primerbank/). *GAPDH* was used for normalization. PCR reactions were using iQ SYBR Green Super mix (Bio-Rad, 170-8882), and performed on iCycler iQ Real-time PCR detection system. The *CSN6* gene copy number was calculated as described(Livak & Schmittgen, 2001). The gene copy numbers as well as the patients' information were analyzed and visualized by Cluster and TreeView software (Eisen, MB), and presented in the form of heat maps.

Primers for Reverse-transcript Quantitative-PCR of *CSN6* (5'-TCATCGAGAGCCCCCTCTTT; 5'- CCAATGCGTTCCGCTTCCT) and p53 target genes, *p21* (5'- CCTGTCACTGTCTTGTACCCT; 5'-GCGTTTGGAGTGGTAGAAATCT), *1433σ* (5'-CTCTCCTGCGAAGAGCGAAAC; 5'- CCTCGTTGCTTTTCTGCTCAA), *BAX* (5'-CCCCGAGAGGTCTTTTTCCG; 5'- GGCGTCCCAAAGTAGGAGA), *PUMA* (5'-GACCTCAACGCACAGTACGAG; 5'-AGGAGTCCCATGATGAGATTGT) were as referred by Primer Bank (http://pga.mgh.harvard.edu/primerbank/). And *GAPDH* was used for normalization. Total RNAs were extracted from cells by TRIZOL (Invitrogen, 15596-018); 1μg RNA was using for producing cDNA by iScript cDNA Synthesis Kit (BioRad, 170-8891). The genes amplification folds were analyzed and visualized by Cluster and TreeView software (Eisen, MB), and presented in the form of heat maps.

Cell Culture and Transfection

Human A549, H1299, U2OS and MCF7 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum; HCT116 cells were maintained in Mcoy's 5A medium supplemented with 10% fetal bovine serum. For transient transfection, cells were transfected with DNA by either Lipofectamine™ 2000 (Invitrogen), or FuGENE® HD (Roche) reagents according to protocols by the manufacturers.

Human breast epithelial cell lines MCF10A, MCF10F, MCF12A were maintained in a 1:1 mixture of DMEM and Ham's F12 supplemented with 5% horse serum, 10 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 0.02 μg/ml epidermal growth factor (Sigma), and antibiotics. The breast cancer cell lines MDA-MB-231, MDA-MB-361, MDA-MB-435, MDA-MB-436, MDA-MB-453, MDA-MB-468, BT-20, BT-474, BT-483, BT-549, MCF7, SkBr3, HBL-100, T47D, Hs578T, ZR-75-1, 11-9-1-4 were maintained in the standard conditions described as ATCC cell culture guidelines.

Generation of Stable Transfectants

For generation of CSN6 knock-down stable cell lines, A549, HCT116, MCF7 and U2OS cells were infected by lentiviral shRNA transduction particles (Sigma, NM_006833 COPS6 MISSION shRNA lentiviral transduction particles) containing either control shRNA or CSN6 shRNA. After infection, cells were selected with 2μg/ml Puromycin for two weeks period according to protocols by the manufacturer.

For generation of Myc-CSN6 overexpression stable transfectants, A549, HCT116, MCF7 and U2OS cells were transfected with either PCDNA6 or PCDNA6-Myc-CSN6 plasmids by

Electroporation (Amaxa). Forty-eight hours after transfection, cells were selected in 8μg/ml Blasticidin containing culture medium for 2 weeks.

Medical Sequencing of p53 gene in Human Breast Cancer Samples

Eighteen primary breast tumor samples were obtained from patients who had undergone operations to treat breast cancer. They were collected as freshly-frozen tissues and were stored in the tissue bank of the University of Texas M.D. Anderson Cancer Center. Genomic DNA was extracted from breast specimens by phenol/chloroform method.

Using PCR amplify entire coding region and the 5' UTR of the *p53* gene locus, subsequent sequencing (Applied Biosystems 3730xl DNA Analyzer) of the PCR products in both directions. The analysis of sequencing results is performed using SeqScape Software (Applied Biosystems) which uses the comparative alignment against a reference sequence to detect mutations. The assay was performed by DNA Analysis Core Facility at the University of Texas M.D. Anderson Cancer Center.

Foci Formation Assay

Microfocus forming assay system was performed as previously described [Reardon, 1993]. Briefly, 1000, 2000 or 3000 cell/ well were plated in 6-well plates for each cell line. Medium was changed every two days over 10 days of foci formation. At the end of the period, cell monolayer was stained in crystal violet solution (0.5% crystal violet, 20% methanol) and then destained by wash with water. Foci were then counted and photographed.

Tumorigenesis in Nude Mice

4-5 weeks old nude mice were maintained in the animal facility at the University of Texas MD Anderson Cancer Center. Mice were randomly divided into experimental groups, five for each. Different stable transfectants of A549 cells (1×10^6) in 0.1 ml PBS per injection were injected subcutaneously into the flanks of mice. Tumor volumes were measured and recorded three times a week from day 5 of the cell inoculation. At the end of 40 days, the mice were sacrificed and the tumors were removed and weighted.

Immunohistochemical Staining of Xenograft Tumors

Different stable transfected A549 cells were injected subcutaneously into the flank regions of female nude mice. Tumor growth was monitored for 40 days. Tumors were isolated at the end of the assay, and fixed with 10% buffered formalin, paraffin embedded, and sectioned.

Sections were stained with anti-p53, anti-MDM2 antibodies and hematoxylin and eosin (H&E) for pathological evaluation.

Immunoprecipitation and Immunoblotting

Total cell lysates were solubilized in lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate, 1 μg each of aprotinin, leupeptin, and pepstatin per ml) and processed as previously described(Laronga et al, 2000). Lysates were immunoprecipitated with indicated antibodies. Proteins were resolved by SDS-PAGE gels; then protein were electrophoretically transferred (Bio-Rad) to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with Blotto buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20, 5% Blotto (Bio-Rad)) for 1 h at room temperature prior to incubation with indicated primary antibodies. Subsequently membranes were washed and incubated for an hour at room temperature with peroxidase-conjugated secondary antibodies (PIERCE). Following several washes, chemiluminescent images of immunodetected bands on the membranes were recorded on X-ray films using the chemiluminescence (ECL) system (Roche Molecular Biochemicals) according to the manufacturer's instructions.

***In vitro* Binding Assay**

The GST-MDM2 (a.a. 1-491, 3-150, 2-290, 384-491, 294-491) fusion constructs were generated by sub-cloning the indicated domains of MDM2 into the pGEX6P-1 GST vector. DNA constructs were transformed into BL21 (DE3) *E.coli*; cells were lysed in the NETN buffer (0.5% NP40, 20mM Tris pH 8.0, 100mM NaCl, 1mM EDTA). Lysates were incubated with GST-beads (Glutathione Sepharose^{TM} 4B, Amersham, 17-0756-01) overnight.

For purifying Flag-CSN6 proteins, the PET-Flag-CSN6 construct was transformed into BL21 (DE3) *E.coli*; cells were lysed in the NETN buffer. Lysates were incubated with M2 Affinity Gel (Sigma, A2220) overnight. The immobilized Flag-CSN6 proteins were eluted by Flag peptide (SIGMA, F3290). The eluted Flag-CSN6 was incubated with different immobilized GST-MDM2 deletions. The retained proteins were resolved by SDS-PAGE and Immunoblotting was performed with anti-Flag antibody.

siRNA

USP2 or USP15 siRNA were obtained from Dharmacon RNAi Technologies, Thermo Scientific. USP15, ON-TARGETplus SMARTpool L-006066-00; USP2, ONTARGETplus SMARTpool L-006069-00.

Gel Filtration

Wild-type or CSN6 siRNA transfected A549 cells were lysed by freeze-thaw in 1ml lysis buffer (KCl 200mM, Hepes 10mM, MgCl2 5mM, CHAPS 0.3%, NaF 50mM, β-glycerol 10mM, DTT 1mM). Lysates were cleared by centrifugation for 30 min, filtered, and fractionated through Superdex 200 column (GE Healthcare) equilibrated with lysis buffer at a flow rate of 0.2 ml/min. Fractions of 200 μl each were collected and subjected to immunoblotting and in vitro ubiquitination assays.

Mass spectrometry analysis

Bacterially purified GST-MDM2 was subjected to in vitro ubiquitination assay, resulted products were separated by SDS-PAGE gel and analyzed by μ-LC/MS/MS mass spectrometry.

(1) In gel digestion

Protein bands were excised and merged from the three independent replicate gels manually, and cut into small pieces. The gel pieces were washed twice with 50 mL of 50% acetonitrile (ACN): 50% 200 mM ammonium bicarbonate for 5 min and shrunk with 100% acetonitrile until the gels turned white; the gels were then dried for 5 min in a speed vac. The gel pieces were rehydrated at room temperature in 15 mL of 50 mM ammonium bicarbonate (37 °C, 4 min). An equivalent volume (15 mL) of trypsin (Promega, Madison, WI) solution (20 ng/mL in 50 mM ammonium bicarbonate) was then added, and the gel pieces were incubated at 37 °C for 4 h or 30 °C for at least 16 h. After digestion, the gel pieces were vortexed and spun down; the resulting supernatant representing the peptide solution was stored at -80 °C until nanoLC/MS/MS analysis.

(2) Liquid Chromatography and Mass Spectrometry

Each digested peptide mixture for LC-MS/MS analyses were introduced into the mass spectrometer via high-performance liquid chromatography using an Agilent (Palo Alto, CA) 1200 series binary HPLC pump and an LC packings FAMOS^{TM} well-plate microautosampler. For each analysis, sample was loaded into a 2 cm × 75 μm i.d. trap column. The trap column was connected to a 11 cm × 75 μm i.d. analytical column and the columns were rigidly packed in-house with 5 μm C_{18} reversed-phase packing material. Mobile phase A consisted of 0.1% formic acid and mobile phase B consisted of 0.1% formic acid in 100% acetonitrile. The flow rate was

200 nl/min. Each reverse-phase step began with 5% acetonitrile for 10 min, a gradient of 5%-40% acetonitrile for 75 min, 40%-85% acetonitrile for 5 min, 85% acetonitrile for 10 min, and then equilibrated with 5% acetonitrile for 20 min. Mass spectrometric analyses were performed on a ThermoFisher Scientific LTQ XL (San Jose, CA) linear ion trap mass spectrometer. A full-mass scan was performed between *m/z* 350 and 2000, followed by MS/MS scans of the five hightest-intensity parent ions at 35% relative collision energy. Dynamic exclusion was enabled with a repeat count of 1, exclusion duration of 3 min, and a repeat duration 30 sec.

(3) Protein identification

The acquired MS/MS spectra were searched against Swissprot protein database 56.8 (release of 10 February 2009) using the Mascot Daemon version 2.2.2. Homo sapiens (human) was chosen for the taxonomic category. Masses for both precursor and fragment ions were treated as monoisotopic. The mass shift of 114.04 Da (GlyGly) or 383.23 Da (LeuArgGlyGly) for lysine residues was included as a variable modification to determine ubiquitination sites. Oxidized methionine, histidine, tryptophan (+ 15.99 Da), carbamidomethylated cysteine (+ 57.02 Da) and deaminated glutamine, asparagines (- 0.98 Da) were also set as variable modifications. Peptide mass tolerance and fragment tolerance were set at 2 Da and 0.5 Da respectively. The initial search was set to allow for up to two missed trypsin cleavages. Decoy database were performed to determine false positive rates. The false positive rates were controlled below 5% by setting p value at 0.025.

Semi-Quantitative RT-PCR

A549 cells were transfected with increasing amount of Myc-CSN6 plasmids. Twenty-four hours later, cells were collected, total RNAs were extracted by TRIZOL (Invitrogen, 15596-018); 1μg RNA was using for producing cDNA by iScript cDNA Synthesis Kit (BioRad, 170-8891). PCR products were analyzed by 0.8% agarose gels.

Luciferase Assay

The p53-responsive element containing BDS2-3X-Luc reporter was transfected with the pCMV-p53 or pCDNA6-myc-*CSN6* expressing vectors into H1299 cells. Luciferase activity was assayed with the dual luciferase assay system (Promega) according to the manufacturer's instructions.

Generation of *Csn6* Knockout Mice

Csn6 locus targeted mouse ES cells (clone number is RRI087) were obtained from BayGenomics. The *Csn6* gene locus was disrupted by insertional mutagenesis. The gene trap vector was used for this cell line is pGT0Lxf, which was inserted into the ninth intron of the *Csn6* gene. After transcription and intron splicing, the exon of the trapped gene abutted the splice acceptor sequence/Engrailed-2 (En2) exon, which is part of the vector. No vector intron sequence was present in mRNA.

C57BL/6 blastocysts were injected with targeted *Csn6* ES cells in the Genetic Engineered Mouse Facility at M. D. Anderson Cancer Center. The injected blastocysts were transferred into pseudo pregnant female recipients and allowed to develop to term. Chimeras born from these surrogate females were raised to maturity prior to mating with wild-type C57BL/6J females (Jackson Lab) in order to test for germ-line transmission. All animals were maintained in the research animal facility of M. D. Anderson Cancer Center. All protocols involving the use of animals were approved by the IACUC committee at M. D. Anderson Cancer Center.

Genotyping

For Southern blot analysis of *NheI*-digested genomic DNA, a 3' probe of 600 bp was generated by PCR with the primers *Csn6*-S1: 5'- CCC GTG ACA CCA ATT CAC – 3' GGA, and *Csn6*-S2: 5' - GTT TGA TGG GGA GCC TAG TGG - 3'. This probe detected a 4.5-kb band in the wild-type allele and a 9-kb band for the targeted allele.

Offspring were genotyped by PCR of tail DNA with the following primers: wild-type allele: forward primer in exon 8 (5' - CCC CAT TCC CCT GCA GTG GCT - 3'), and reverse primer in exon 10 (5' - AAA CAG TCC CCG CAT TCG CCG - 3'); mutant allele: forward primer in *lacZ* (5' - GCA TCG AGC TGG GTA ATA AGC GTT GGC AAT - 3'), reverse primer in *lacZ* (5' - GAC ACC AGA CCA ACT GGT AAT GGT AGC GAC - 3'). For embryo genotyping, the embryos were dissected and soaked in 15-20μl PCR buffer with 1% proteinase K and 1% Triton X-100 at 55°C for 2 h. After heat inactivation of proteinase K, 3-5 μl of the solution was used for the PCR reaction to determine the genotype.

Mouse Organ Extracts

Brain, lung, heart, thymus, liver, spleen, kidney, and skeletal muscle were isolated from age-matched littermates and immediately submerged in liquid nitrogen. The solid organs were ground and lysed in lysis buffer with brief sonication. Protein samples were resolved on 10% SDS-PAGE gel.

***In vivo* Ubiquitination Assay in MEF Cells**

The wild-type or *CSN6*$^{+/-}$ cells were treated with 50 μg/ml MG132 for 6 h before harvest. Cells were lysed with lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP40, 0.5% Triton X-100, 5 mM NEM). The endogenous p53 proteins were immunoprecipitated by the monoclonal anti-p53 antibody, DO-1 and the endogenous MDM2 proteins were immunoprecipitated with anti-MDM2 antibody, OP115T. The protein complexes were then resolved by 6% SDS-polyacrylamide gel and probed with anti-Ubiquitin to observe the ubiquitinated p53 or MDM2.

Quantitative RT-PCR

Primary MEF cells from same littermates with different genotypes were treated with doxorubicin (1μg/ml) for 18hrs or without treatment. Cells were collected, total RNAs were extracted by TRIZOL (Invitrogen, 15596-018); 1μg RNA was using for producing cDNA by iScript cDNA Synthesis Kit (BioRad, 170-8891). Primers for Reverse-transcript Quantitative-PCR of mouse *p21* are 5'- GGAGGACCACGTGGCCTTGT; 5'- AATCTGTCAGGCTGGTCTGC; for mouse *Csn6* are 5'- TGATTGGGGCTCTGATCGG; 5'- CAACCCAGAAACTCCAGCTCC.

Generation of *Csn6*/*p53* Double Knockout Mice

p53$^{+/-}$ mice in C57BL/6J background were obtained from Dr. Guillermina Lozano. In order to obtain *Csn6/p53* double knockout mice, timed cross was set between male-*Csn6*$^{+/-}$; *p53*$^{-/-}$ mice and female-*Csn6*$^{+/-}$; *p53*$^{+/-}$ mice.

TUNEL Assay

Five-week-old mice of both sexes and genotypes (*Csn6*$^{+/+}$=4, *Csn6*$^{+/-}$=5) were treated with 5-Gy TBI and sacrificed 5 h later. Harvested thymuses were fixed in phosphate-buffered formalin for 24 h. They were processed, embedded in paraffin and sectioned. The TUNEL (Promega, DeadEnd Colorimetric TUNEL System, G7360) staining on the slides was performed according to the manufacturer's instructions.

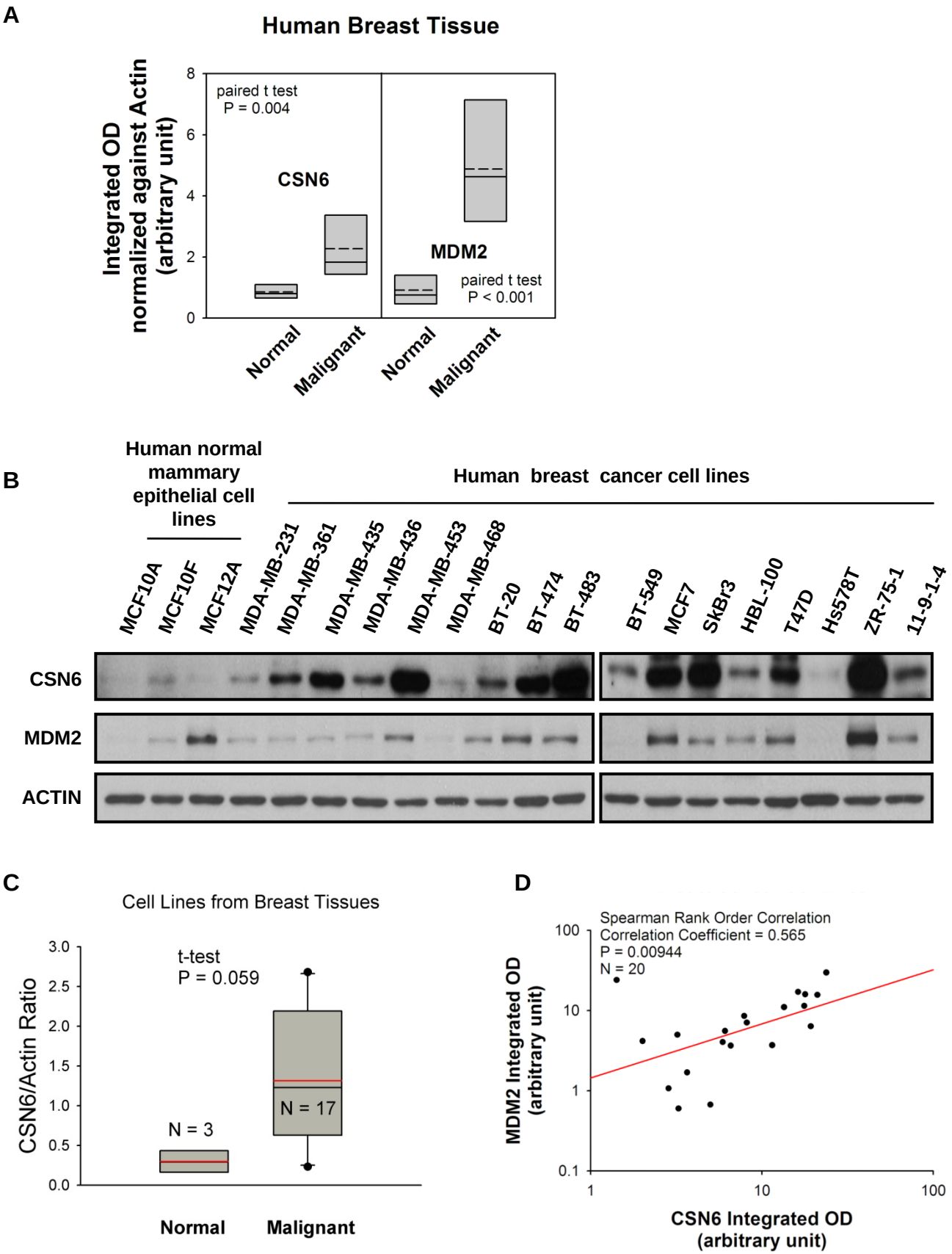
Cleaved Caspase-3 Staining

Pregnant females were treated at 13.5 days post-coitus with 5-Gy TBI and sacrificed 5 h later to collect embryos. Embryos (*Csn6*$^{+/+}$=5, *Csn6*$^{+/-}$=4) were fixed in phosphate-buffered formalin for 24 h. They were processed, embedded and sectioned. Cleaved caspase-3 (Biocare Medical LLC) staining was performed with the assistance of the Histology Core Lab of M.D. Anderson Cancer Center.

Survival Analysis

Five-week-old mice of both sexes and genotypes (*Csn6*$^{+/+}$=13, *Csn6*$^{+/-}$=10) were exposed to a single dose of 7.5 Gy TBI and observed daily. Moribund animals were euthanized. The survival curves were analyzed by the Kaplan-Meier method and compared using the log-rank test.

Supplemental Figure 1



Supplemental Figure 1. Expression of CSN6 or MDM2 in human breast tissues.

(A) The bands on the immunoblots of human breast tissue samples shown in Figure 2A were measured using a densitometer. The box plots of the ratios of CSN6/actin expression and the ratios of MDM2/actin expression in normal breast tissue and in breast cancer samples are shown.

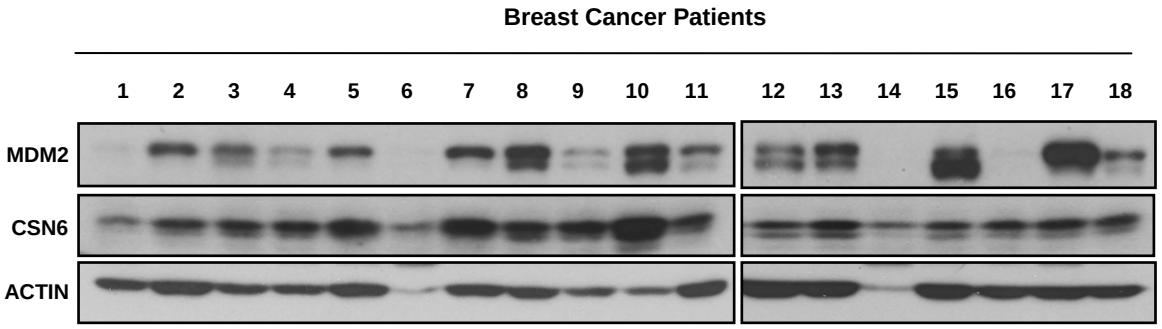
(B) The CSN6 and MDM2 expression levels in non-malignant breast cell lines and breast cancer cell lines were analyzed by immunoblotting. The immunoblots are shown as labeled with the antigen proteins on the left.

(C) Levels of CSN6 and actin in each cell line in (B) were measured using a densitometer. The box plots of the ratios of CSN6/actin expression in non-malignant breast cell lines and in breast cancer cell lines are shown.

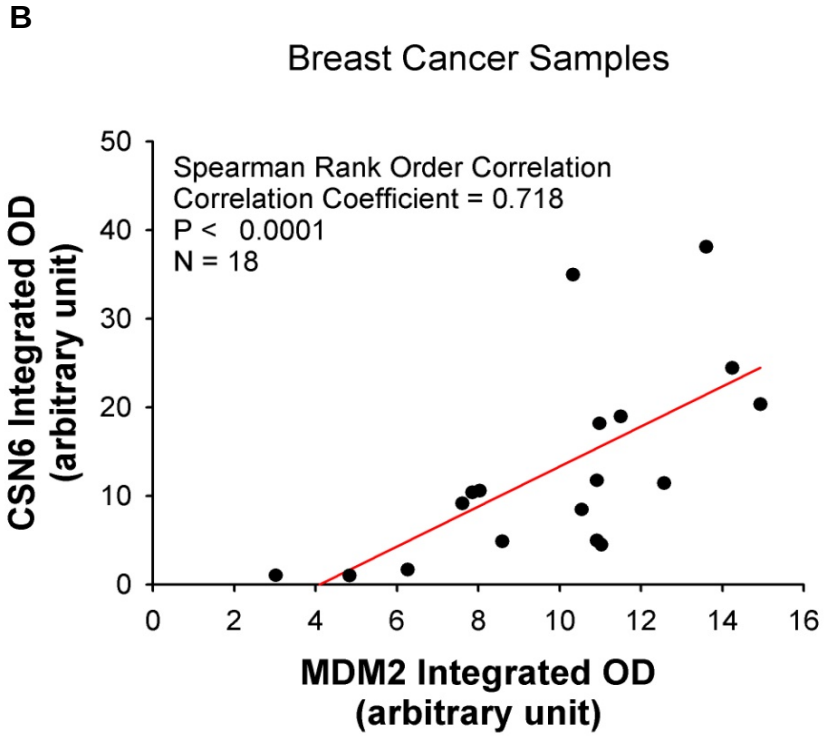
(D) The integrated optical density (OD) of MDM2 was plotted against that of CSN6. Spearman's rank order correlation was used to demonstrate the positive correlation between CSN6 and MDM2 protein levels.

Supplemental Figure 2

A



B

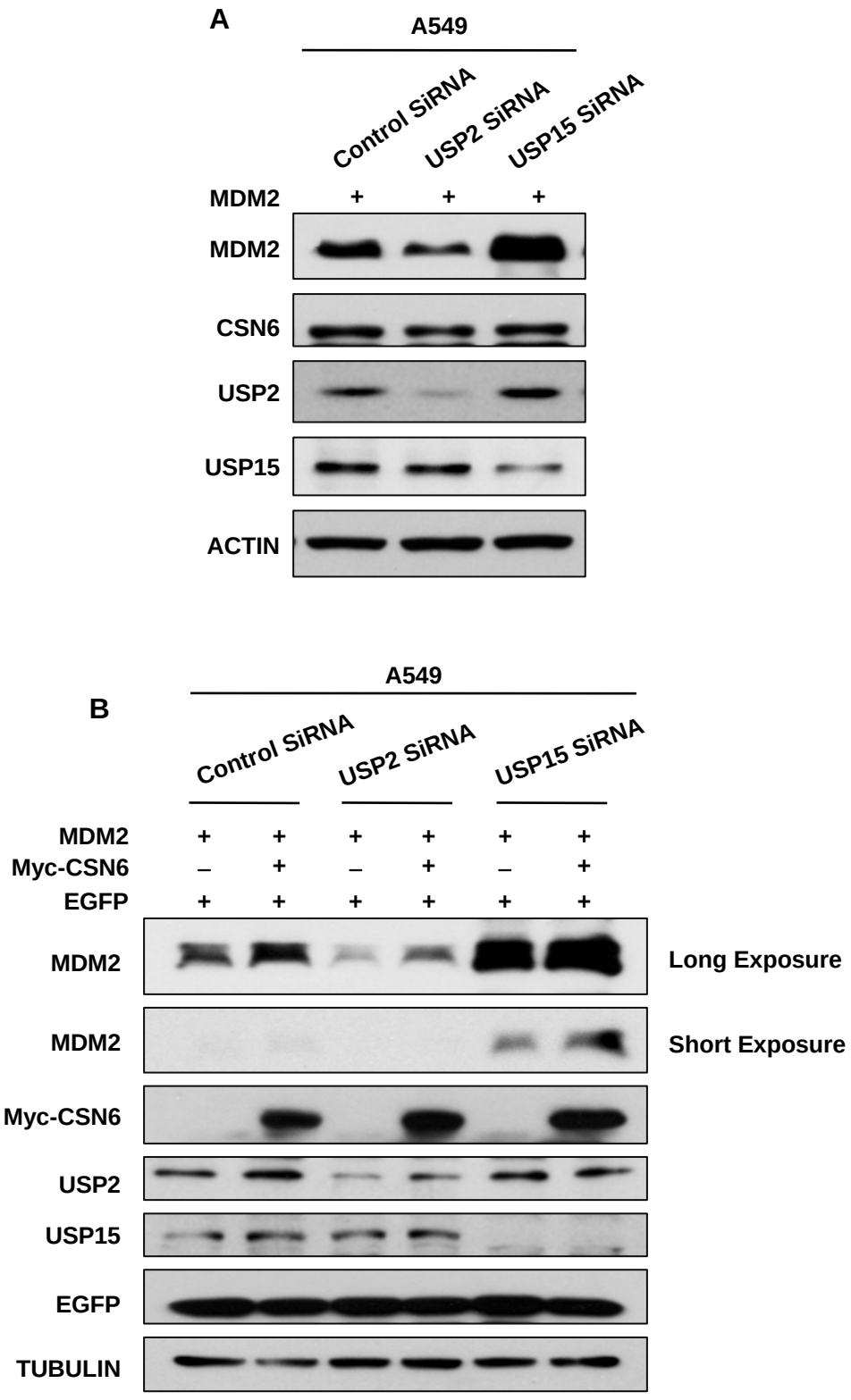


Supplemental Figure 2. Expression of CSN6 or MDM2 in malignant human breast cancer specimens.

(A) The CSN6 and MDM2 expression levels in malignant human breast cancer specimens were analyzed by immunoblotting. The immunoblots are shown as labeled with the antigen proteins on the left.

(B) The integrated optical density (OD) of MDM2 from (A) was plotted against that of CSN6. Spearman's rank order correlation was used to demonstrate the positive correlation between CSN6 and MDM2 protein levels.

Supplemental Figure 3

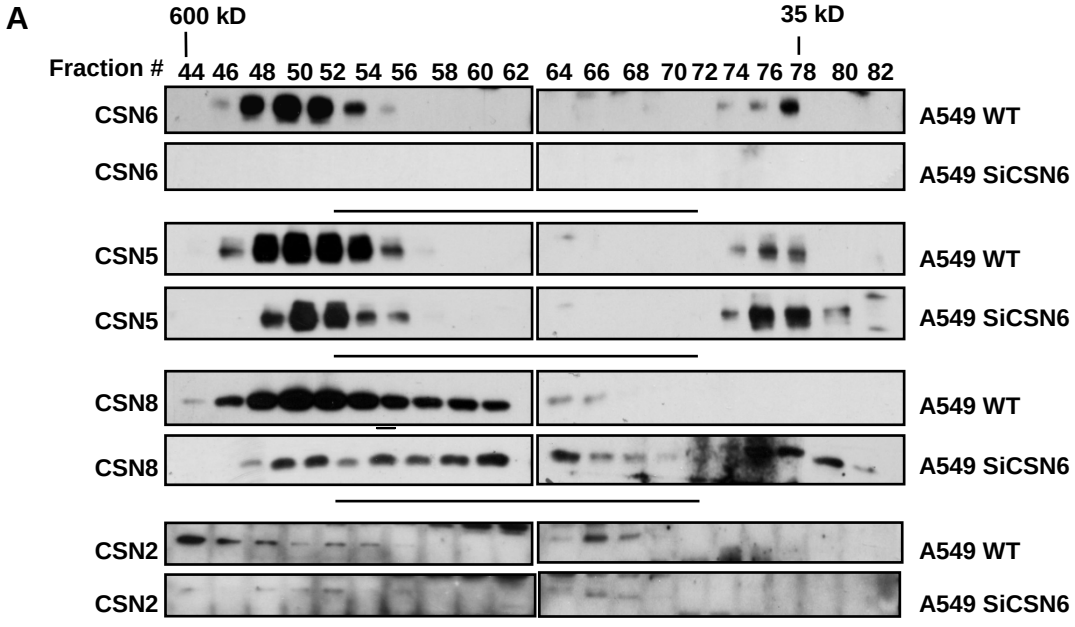


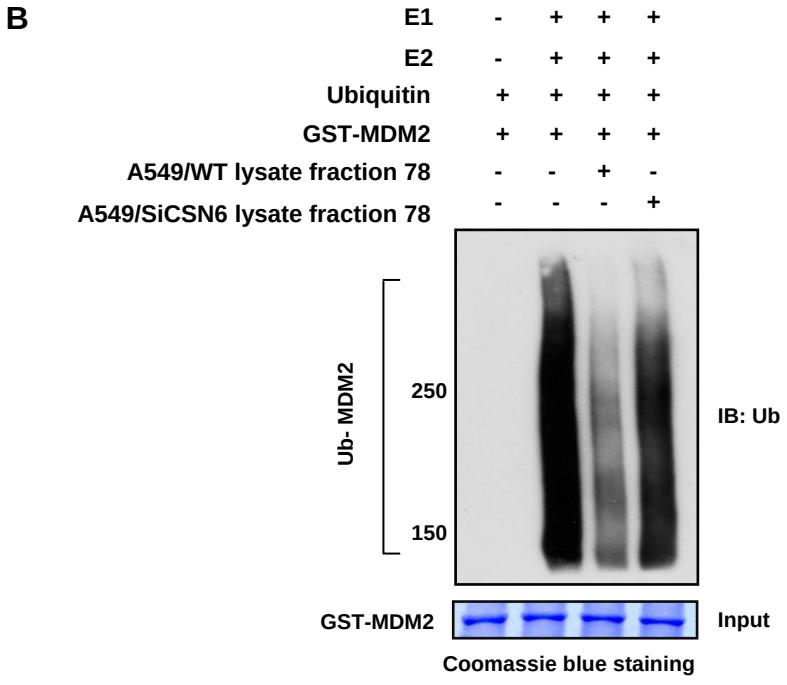
Supplemental Figure 3. USP2 and USP15 are not involved in the regulation of CSN6 on MDM2.

(A) USP2 and USP15 showed different impact on MDM2 protein stability. USP2 or USP15 siRNA (Dharmacon RNAi Technologies, Thermo Scientific. USP15, ON-TARGETplus SMARTpool L-006066-00; USP2, ON-TARGETplus SMARTpool L-006069-00) were transfected into A549 cells; 48-hour after the siRNA transfection, MDM2 expression vectors were introduced into the same cells. Thirty hours after plasmids transfection, cells were collected and lysates were subjected to indicate immunoblotting.

(B) CSN6 stabilizes MDM2 is independent of USP2 and USP15. USP2 or USP15 siRNA were transfected into A549 cells; 48-hour after the siRNA transfection, MDM2, Myc-CSN6 and EGFP expression vectors were introduced into the same cells as shown. Thirty hours after plasmids transfection, cells were collected and lysates were subjected to indicate immunoblotting. EGFP served as a transfection efficiency control as well as gel loading control.

Supplemental Figure 4



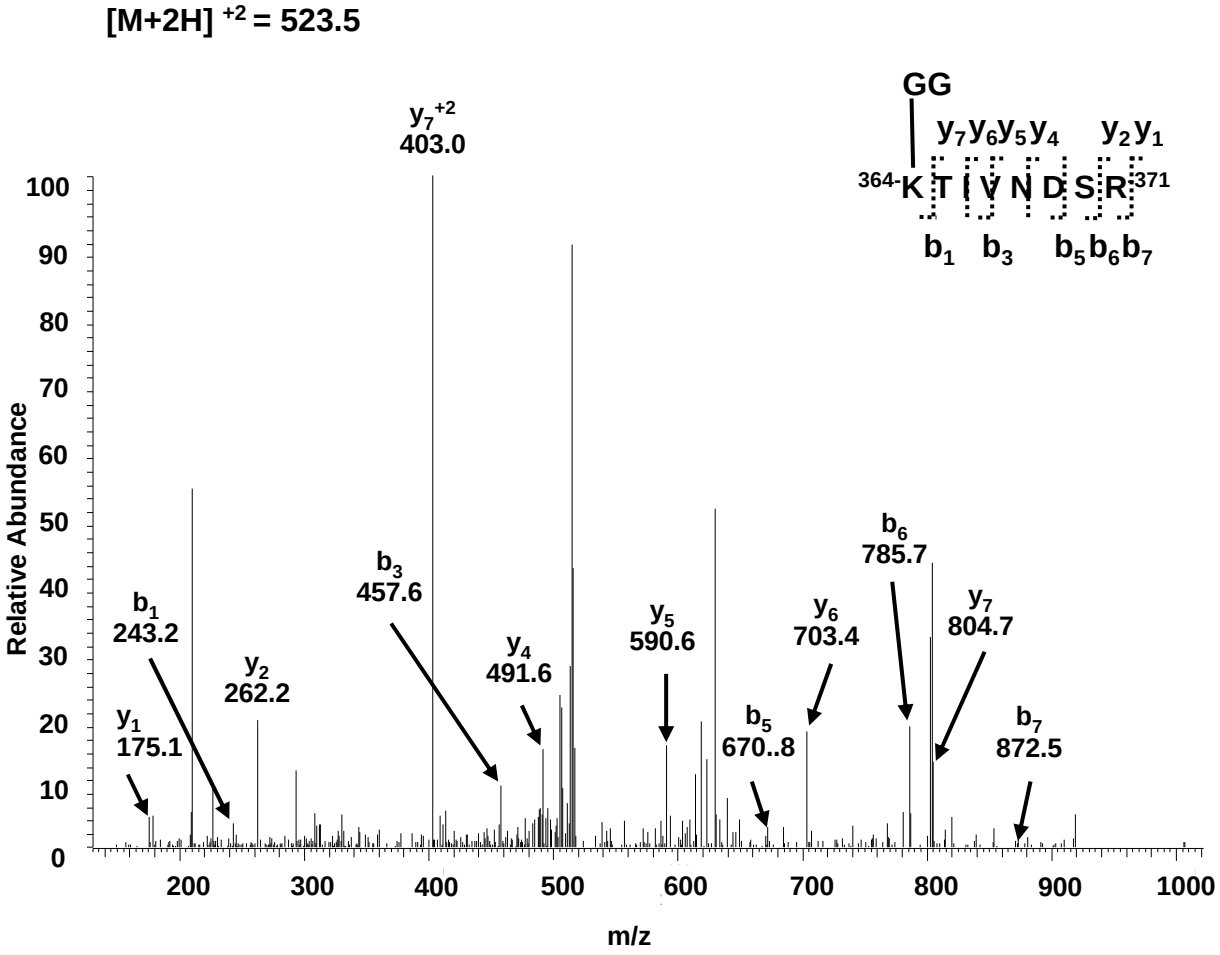


Supplemental Figure 4. Free form of CSN6 exists inside the A549 cells and is required for the inhibition of poly-ubiquitination of MDM2.

(A) Free form of CSN6 exists inside the A549 cells. Extracts of A549 cells (wild-type or transfected with CSN6 siRNA for 72 hours) were fractionated by gel filtration chromatography. An aliquot of each fraction was immunoblotted with antibodies to CSN6, CSN5, CSN8 and CSN2 to detect the distribution profiles of CSN complex as well as each subunit. Only CSN6 and CSN5 show the existence of free form. There is no detectable CSN6 signal in both holo-complex (fraction # 46-56) and free form (fraction #74-78) when depleted CSN6 by specific CSN6 siRNA. Moreover, the amounts of free form CSN5 and CSN8 increased upon knockdown of CSN6, the amounts of CSN2 and CSN holo-complex decreased after knockdown of CSN6.

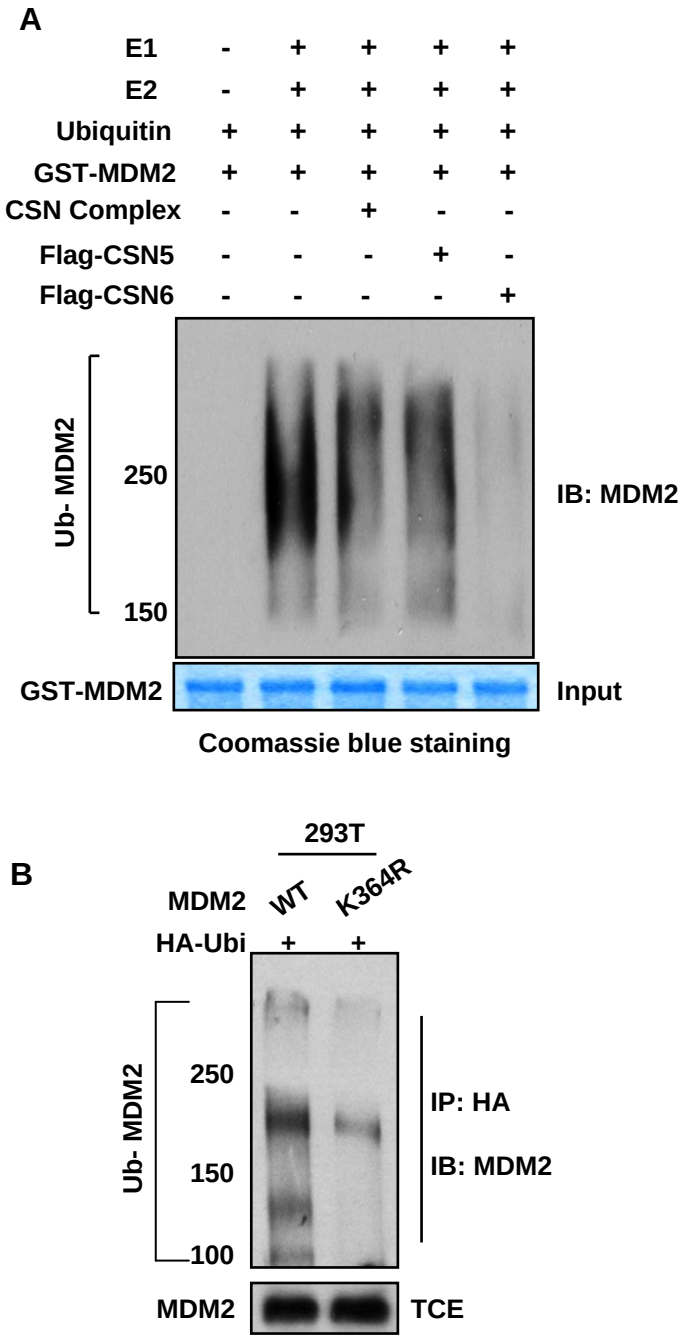
(B) CSN6 is required for the inhibition of poly-ubiquitination of MDM2 in an *in vitro* ubiquitination assay. CSN6 Free form containing fraction (#78) from either wild-type or CSN6 knockdown A549 cell extracts were used for in vitro ubiquitination assay. GST-MDM2 was incubated with different fractions in the presence of E1, E2, His-Ubiquitin, and ATP as indicated. The ubiquitinated MDM2 was detected by anti-Ubiquitin.

Ubiquitination modification site : Lys 364



Supplemental Figure 5. Identification of MDM2 auto-ubiquitination site by mass spectrometry analysis.

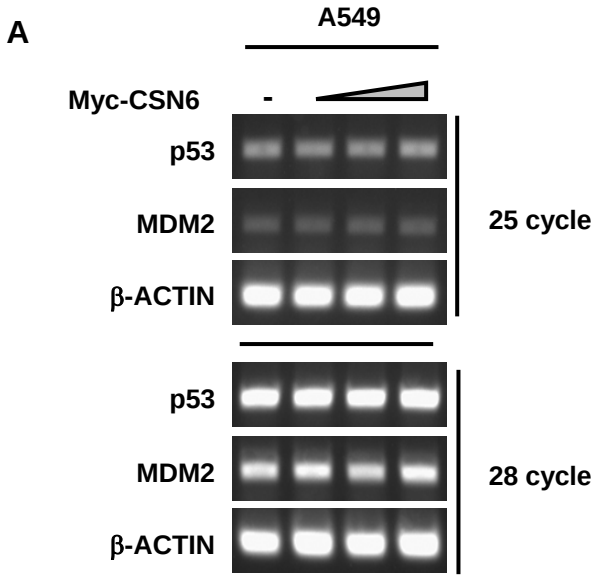
In vitro ubiquitination products (samples prepared procedure as in Figure 4F) of GST-MDM2 were subjected to mass spectrometry analysis and Lys 364 was identified as the auto-ubiquitination site of MDM2.

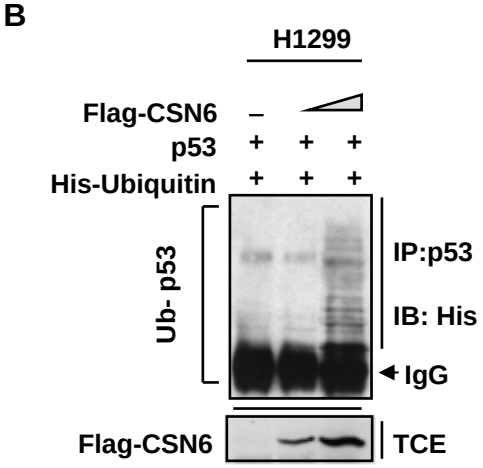


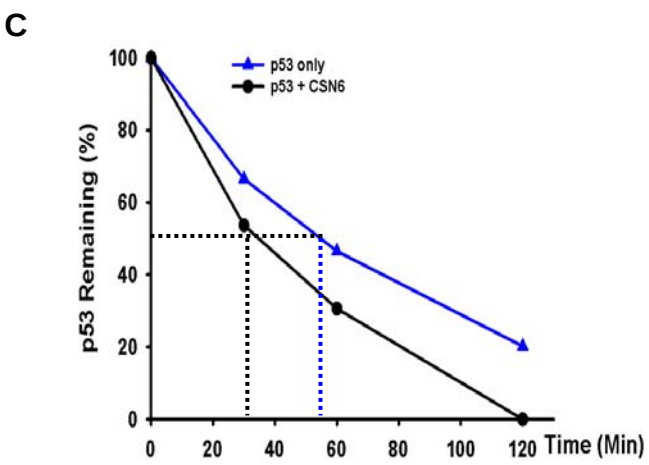
Supplemental Figure 6. CSN6 inhibits auto-ubiquitination of MDM2 at Lys364.

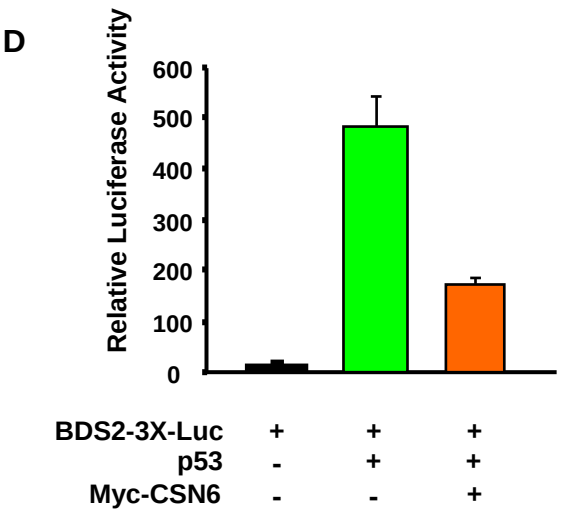
(A) CSN6 reduced the ubiquitination level of MDM2 in an *in vitro* ubiquitination assay. The samples as described in Figure 4F were blotted by anti-MDM2 antibody to examine poly-ubiquitination of MDM2.

(B) The abolishment of Lys364 of MDM2 attenuates the auto-ubiquitination of MDM2 in vivo. Wild-type or K364R mutation form of MDM2 were transfected to 293T cells with equal amount of HA-Ubiquitin. Cells were treated with MG132 for 6 hr after 24 hr transfection. In vivo ubiquitination assay was performed to identify the auto-ubiquitination levels of MDM2.


A
A549
Myc-CSN6
-
p53
MDM2
β-ACTIN
25 cycle
p53
MDM2
β-ACTIN
28 cycle


B
H1299
Flag-CSN6
p53
His-Ubiquitin
Ub- p53
IP:p53
IB: His
IgG
Flag-CSN6
TCE


C
p53 only
p53 + CSN6
p53 Remaining (%)
Time (Min)


D
Relative Luciferase Activity
BDS2-3X-Luc
p53
Myc-CSN6

Supplemental Figure 7. Overexpression of CSN6 inhibited p53 transcription activity.

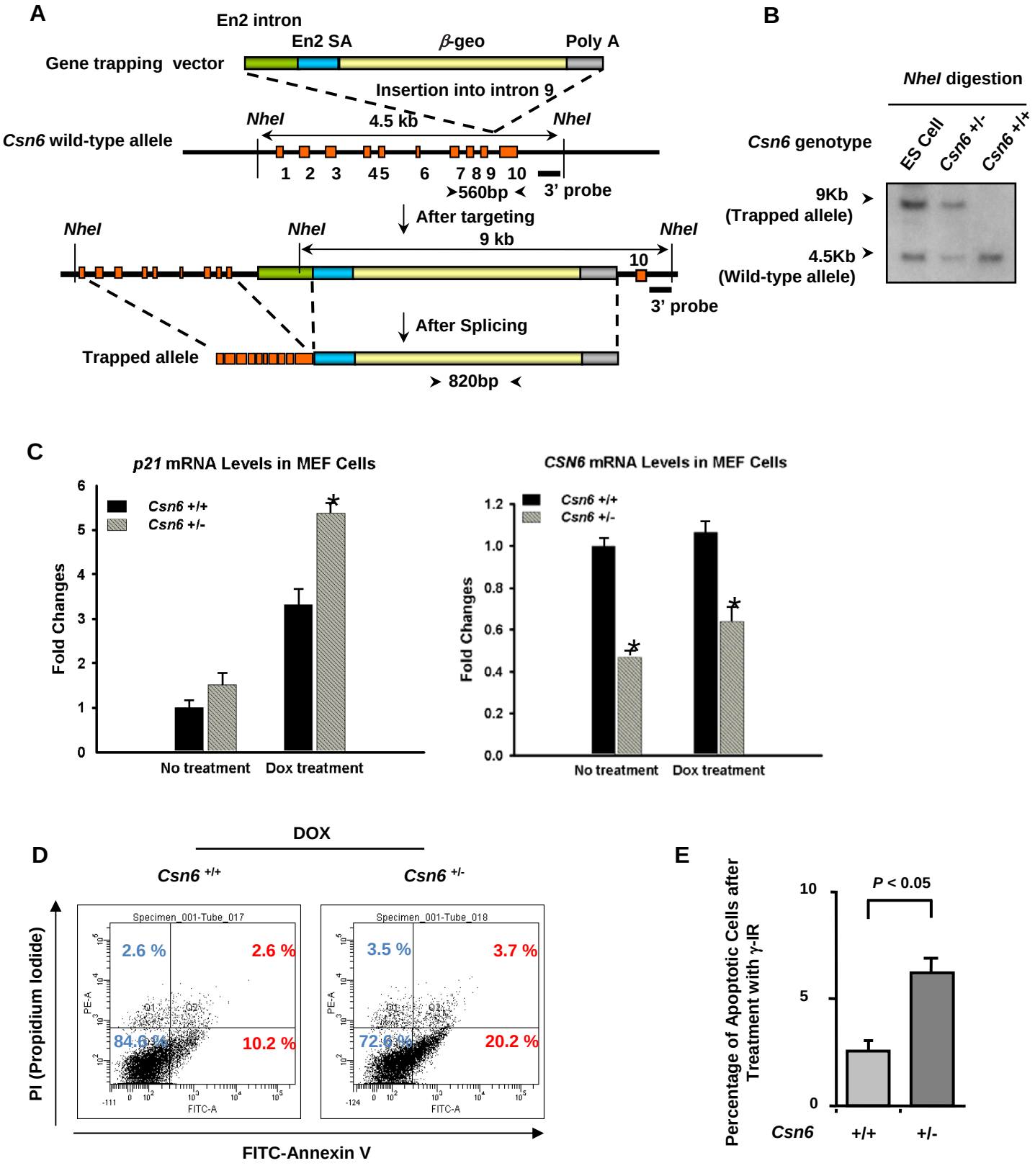
(A) The mRNA levels of p53 and MDM2 were not affected by the increased expression of CSN6. Semi-quantitative RT-PCR analysis of p53, MDM2 and actin in A549 cells transfected with increasing amount of Myc-CSN6 showed no obvious difference in either p53 or MDM2 transcription. Representative picture was the PCR product which cycles were still within the linear range of amplification.

(B) CSN6 enhances poly-ubiquitination of p53. H1299 cells were cotransfected with pCMV-p53, pCMV-His-ubiquitin and increasing amounts of pCMV-Flag-CSN6. The cells were treated with MG132 for 6 h before harvest, and the His-ubiquitinated p53 was immunoprecipitated with anti-p53 and probed with anti-His antibody. Equal amounts of TCE were immunoblotted with anti-Flag.

(C) Integrated OD values of p53 in Fig 5F at each time point were measured and the level of p53 at time 0 was set at 100%. p53 remaining is indicated graphically.

(D) Enforced expression of CSN6 impairs p53 transcriptional activation determined by BDS2-3X-Luc (containing p53 response element) reporter activity in H1299 cells.

Supplemental Figure 8



Supplemental Figure 8. Depletion of *Csn6* activated the function of p53.

(A) Diagram of the *Csn6* targeted allele in mouse ES cells. The β-*geo* [β-galactosidase gene (*LacZ*) and neomycin-resistance] fusion gene-containing gene-trap vector (8.6 kb) was inserted at intron 9 of the *Csn6* gene. The location of the 3' probe used for Southern blot is indicated. Arrow heads, primers used in (Figure 5A) and the expected size of the PCR product is shown. En2, *Engril 2*; SA, splice acceptor.

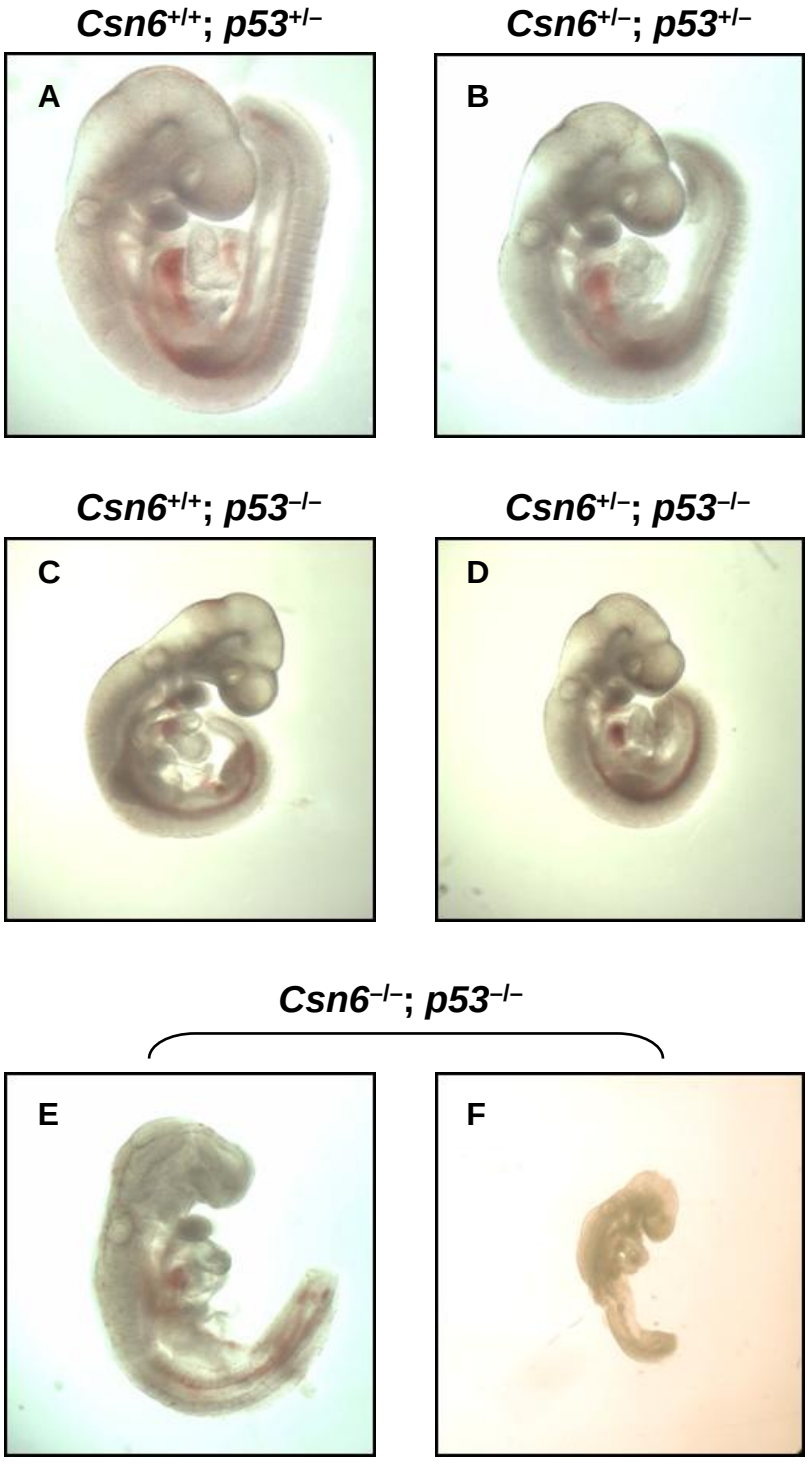
(B) Genotyping of progenies using Southern blot analysis. Genomic DNA extracted from progenies was digested with *NheI* restriction enzyme. 3' probe detected the wild-type allele (4.5 kb) and the trapped allele (9 kb).

(C) CSN6 haplo-insufficiency upregulated the expression of p21 mRNA in MEF cells. Primary $Csn6^{-/-}$ or $Csn6^{+/+}$ MEF cells were treated with doxorubicin (1μg/ml) for 18hrs, and the expression of *Csn6* and p53-dependent gene *p21* were assessed by RT-q-PCR. Folds of change of *p21* mRNA or *Csn6 mRNA* expression are represented by bar graphs, * means $p<0.05$; error bars, 95% confidence interval. Data are from typical experiments conducted in triplicate.

(D) Csn6 haplo-insufficiency sensitized MEF cells to doxorubicin-induced apoptosis. Primary $Csn6^{+/-}$ or $Csn6^{+/+}$ MEF cells were treated with 1μg/ml DOX for 48 h or without treatment. Cells were harvested and analyzed with Annexin-V and propidium iodide staining, followed by FACS analysis.

(E) CSN6 haplo-insufficiency sensitized MEF cells to γ-IR-induced apoptosis. Primary $Csn6^{+/-}$ or $Csn6^{+/+}$ MEF cells were treated with γ-IR at 10 Gy or without treatment. Forty-eight hours later, cells were harvested and analyzed with Annexin-V and propidium iodide staining, followed by FACS analysis. Percentages of apoptotic population in $Csn6^{+/+}$ and $Csn6^{+/-}$ MEFs were measured and indicated graphically, two-tailed Student's t-test, $p< 0.05$.

Supplemental Figure 9



Supplemental Figure 9. The embryonic lethality of *Csn6*-null mice can be partially rescued by concomitant loss of p53.

Representative E9.5 embryos (with genotypes indicated) from intercross between male *Csn6*$^{+/-}$ *p53*$^{-/-}$ mice and female *Csn6*$^{+/-}$*p53*$^{+/-}$ mice are shown. *Csn6*$^{-/-}$*p53*$^{+/+}$ embryos cannot be detected at E9.5 (see Table 1), but *Csn6*$^{-/-}$*p53*$^{-/-}$ embryos can be identified at this embryonic stage. Note that *Csn6*$^{-/-}$*p53*$^{-/-}$ embryos were less developed (panel E) or much smaller (panel F) when compared to other embryos.

Supplemental Table 1

CSN6, *MDM2* and *p53* Gene Statuses of Breast Cancer Patient Samples*

Specimen	CSN6 Gene Amplification	MDM2 Gene Amplification	p53 Gene Status		
			Status	DNA Base Change	Amino Acid Change
Patient 2	No	No	Heterozygous missense mutation	1. Exon 4, 119 C→ C/G, SNP (rs 1042522). 2. Exon 5, 98 G → G/A, missense mutation. 3. Exon 8, 6 G → G/T, missense mutaion	1. Exon,4, P 72 → P/R (SNP) 2. Exon 5, R 158→ H/R. 3. Exon 8, G 262 → G/V
Patient 3	Yes	No	Wild-type	Exon 4, 119 C→ G, SNP (rs 1042522).	Exon,4, P 72 → R (SNP)
Patient 5	Yes	No	Wild-type	Exon 4, 119 C→ G, SNP (rs 1042522).	Exon,4, P 72 → R (SNP)
Patient 7	Yes	No	Wild-type	Exon 4, 119 C→ G, SNP (rs 1042522).	Exon,4, P 72 → R (SNP)
Patient 8	No	No	Wild-type	Non-coding region change is not indicated.	
Patient 10	Yes	No	Wild-type	Exon 4, 119 C→ C/G, SNP (rs 1042522).	Exon,4, P 72 → P/R (SNP)
Patient 13	Yes	Yes	Heterozygous deletion mutation	Exon 7, [51-51 del C] + [C].	Heterozygous deletion mutation start from S 242.
Patient 15	No	Yes	Wild-type	Non-coding region change is not indicated.	
Patient 17	Yes	No	Wild-type	Exon 4, 119 C→ C/G, SNP (rs 1042522).	Exon,4, P 72 → P/R (SNP)

*** Samples in Figure S2A with positive correlation of CSN6 and MDM2 protein levels were selected for genomic DNA sequencing. Total nine out of eighteen samples were examined.**

Supplemental Table 2

Genetic Analysis and Offspring Number of Intercross Between Double-Heterozygous Mice (*Csn6*$^{+/-}$; *p53*$^{+/-}$ and *Csn6*$^{+/-}$; *p53*$^{+/-}$)

Potential Genotypes and Expected Ratio of Offspring	Actual Identified Number and Ratio
Csn6$^{+/+}$; ***p53***$^{+/+}$, (1/16=6.25%)	**23** (9.8%)
Csn6$^{+/+}$; ***p53***$^{+/-}$, (2/16=12.5%)	**39** (16.7%)
Csn6$^{+/+}$; ***p53***$^{-/-}$, (1/16=6.25%)	**15** (6.4%) (Male: 9; Female: 6)
Csn6$^{+/-}$; ***p53***$^{+/+}$, (2/16=12.5%)	**47** (20.1%)
Csn6$^{+/-}$; ***p53***$^{+/-}$, (4/16=25%)	**85** (36.3%)
Csn6$^{+/-}$; ***p53***$^{-/-}$, (2/16=12.5%)	**25** (Male: 19; Female: 6) (10.7%)
Csn6$^{-/-}$; ***p53***$^{+/+}$, (1/16=6.25%)	**0**
Csn6$^{-/-}$; ***p53***$^{+/-}$, (2/16=12.5%)	**0**
Csn6$^{-/-}$; ***p53***$^{-/-}$, (1/16=6.25%)	**0**
Total identified live birth litter numbers	**234** (Male:141; Female: 93)