PNAS
www.pnas.org

Supplementary Information for

ACTL6A promotes repair of cisplatin-induced DNA damage, a new mechanism of platinum resistance in cancer

Yang Xiao, Fang-Tsyr Lin and Weei-Chin Lin

Corresponding author:
Weei-Chin Lin
Email: weeichil@bcm.edu

This PDF file includes:

SI Materials and Methods

Figures S1 to S4

SI References

SI Materials and Methods

Recombinant plasmids and shRNA expression vectors

Retroviral pQCXIP vector was used for the cloning of HA-tagged mouse ACTL6A cDNA. Briefly, the full-length ACTL6A cDNA sequences were amplified by polymerase chain reaction (PCR) using mouse embryonic fibroblast cDNA as the template. The primers 5'ATTAGCGGCCGCCCACCATGTACCCATACGATGTTCCAGATTACGCTAGCGGCGG CGTGTACGGCGGAGATGAA-3' and 5'-ACGCTTAATTAAGCTACAGACGTTGAGGTGACGGGCA-3' were used for amplification, such that the HA tag was inserted at the N-terminus of the protein. The PCR product was digested and cloned into the Not I/Pac I sites of pQCXIP vector, and was verified by DNA sequencing. The dominant-negative constructs of Brg1 and Brm were kindly provided by Dr. Weidong Wang (1) and Dr. Christian Muchardt (2), respectively. The expression vector of mouse ACTL6A M3 mutant (E388A/R389A/R390A) was kindly provided by Dr. Sunny (Songyang) Zhou (3). The pGIPZ lentiviral expression vectors of human ACTL6A shRNA (V2LHS_15611 (#1), V2LHS_15612 (#2), and V2LHS_15613 (#3)) and a scrambled shRNA control lentiviral vector were purchased from Open Biosystems, Inc. The pLKO.1-puro lentiviral expression vectors of validated human BAF155 shRNA (shBAF155_#1, TRCN0000015628; shBAF155_#2, TRCN0000015630), INO80 shRNA (shINO80_#1, TRCN0000107556; shINO80_#2, TRCN0000107558), and TIP60 shRNA (shTIP60_#1, TRCN0000020315; shTIP60_#2, TRCN0000020318) were purchased from Sigma-Aldrich. The pLKO.1-puro lentiviral expression vector of a scrambled shRNA (4) was obtained from Addgene.

Cell lines, cell infection and transfection

A2780cis cell line (ECACC 93112517) (5, 6) was purchased from PHE-Culture collections through Sigma. Other human cell lines were purchased from ATCC. Human lung cancer cells A549, H460, H520, H358, H125 and H226 were grown in RPMI 1640 medium. HEK293T, H1299 and A2780cis cells were grown in Dulbecco's modified Eagle's medium (DMEM). Both media were supplemented with 10% fetal bovine serum (FBS), penicillin (50 IU/ml), and streptomycin (50 μg/ml) in a humidified incubator with 5% CO2 at 37°C. Both retrovirus and lentivirus expressing indicated proteins or shRNA were produced in HEK293T cells. Twenty-four to thirty-six hours after transfection, the virus-containing medium was collected to infect cancer cells with the addition of 8 μg/ml Polybrene. Two days after infection, the virus-containing medium was replaced with fresh culture medium. To establish stable cell lines, infected cells were selected with 1-3 μg/ml puromycin for one week, and the indicated protein expression was confirmed by immunoblotting. For transient transfection, the plasmid DNA was delivered into cells using PolyJet *in vitro* DNA transfection reagent (SignaGen). Thirty-six to forty-eight hours after transfection, cells were treated or harvested for the analysis.

Cell viability and clonogenic survival assays

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability. Cells were seeded in 96-well plates at 5,000-10,000 cells per well for 24 h, followed by indicated treatment for another 48 h. Cells were then washed and incubated with MTT solution (Thiazolyl blue tetrazolium bromide, 5 mg/ml) (Sigma) at 37℃ for 3-4 h. After the removal of medium and MTT solution, 100 μl dimethylsulfoxide (DMSO) was added to each well. The absorbance was read at 570 nm on a plate reader (BioTek Synergy HT). Each experiment was performed at least in triplicates. For colonogenic survival assay, cells were seeded in 6-well plates at 300-500 cells per well. Within 24 h after seeding, cells were treated with the indicated drugs for

24 h. Cells were then washed twice with PBS and incubated in fresh culture medium for 8-12 days. The colonies were fixed with 4% formaldehyde, and then stained with 0.5% crystal violet in 25% methanol solution. Plates were scanned and the colonies containing more than 50 cells were counted.

Caspase activity assay

The A2780cis stable cells harboring either a scrambled shRNA (shScr) or an ACTL6A shRNA (shACTL6A#2 or #3) were treated with cisplatin (20 μM or 40 μM) for 20 h. Cells were then harvested and apoptosis was determined by the Caspase-Glo® 3/7 activity assay according to the manufactural instruction (Promega). The caspase-3/7 activity was determined by cleavage of the luminogenic substrate containing the DEVD sequence, and was normalized to protein concentrations.

Western blotting and immunoprecipitation

Cells were lysed in TNN buffer (50 mM Tris, 0.25 M NaCl, 5 mM EDTA, and 0.5% Nonidet P-40), and total proteins of tumor samples were extracted in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) with protease inhibitor cocktails. The lysates were then sonicated as described previously (7, 8). To detect cellular co-immunoprecipitation, cell lysates were incubated with control IgG or indicated antibodies overnight and then with protein A/G agarose beads (GenDEPOT) for 1 hour at 4°C, followed by washing three times with TNN buffer. To immunoprecipitate HA-tagged proteins, cell lysates were incubated with anti-HA monoclonal antibody-conjugated agarose beads (Sigma) overnight at 4°C, and were then washed three times in TNN buffer. All the samples were boiled in SDS loading buffer, separated by SDS-PAGE and electrotransferred to the polyvinylidene fluoride (PVDF)

membrane (Millipore). After blocking with 5% milk, immunoblotting was performed with the appropriate antibodies. The antibody specific to ACTL6A (E3), GAPDH (6C5), Brm (E1), TIP60 (3557C4a) or HA-tag (Y11) was purchased from Santa Cruz Biotechnology. The antibody specific to γH2AX (20E3), Brg1 (D2Q7F) or Brm (D9E8B) was purchased from Cell Signaling Technology. Anti-H2AX and anti-acetyl Histone H4K16 antibodies were purchased from Millipore. Anti-acetyl Histone H3 antibody was purchased from Upstate (Millipore, 06-599). The antibody specific to Histone H4, acetyl Histone H3K18, or acetyl Histone H4K8 was purchased from Abcam. The antibody specific to BAF155, BAF170 or INO80 was purchased from BETHYL Laboratories.

RNA extraction and Quantitative real-time PCR

RNA was extracted from different lung cancer cell lines using TRIzol reagent (Invitrogen), and Quantitative PCR was performed in triplicates on MX3005P thermal cycler using SYBR Q-PCR Master Mix (GenDEPOT). The primer pairs used for ACTL6A amplification are 5'-TGGAGGCCATTTCACCTCTAA-3' and 5'-TCTTTGCTCTAGTATTCCACGGT-3', and the primer pairs used for GAPDH amplification are 5'-ATTGGGCGCCTGGTCACCAGGGCTG-3' and 5'-AAATGAGCCCCAGCCTTCTCCATG-3'. The ACTL6A mRNA levels were analyzed with MxPro 4.0 quantitative PCR (QPCR) software (Stratagene), and were normalized to that of GAPDH.

***In vivo* xenograft model**

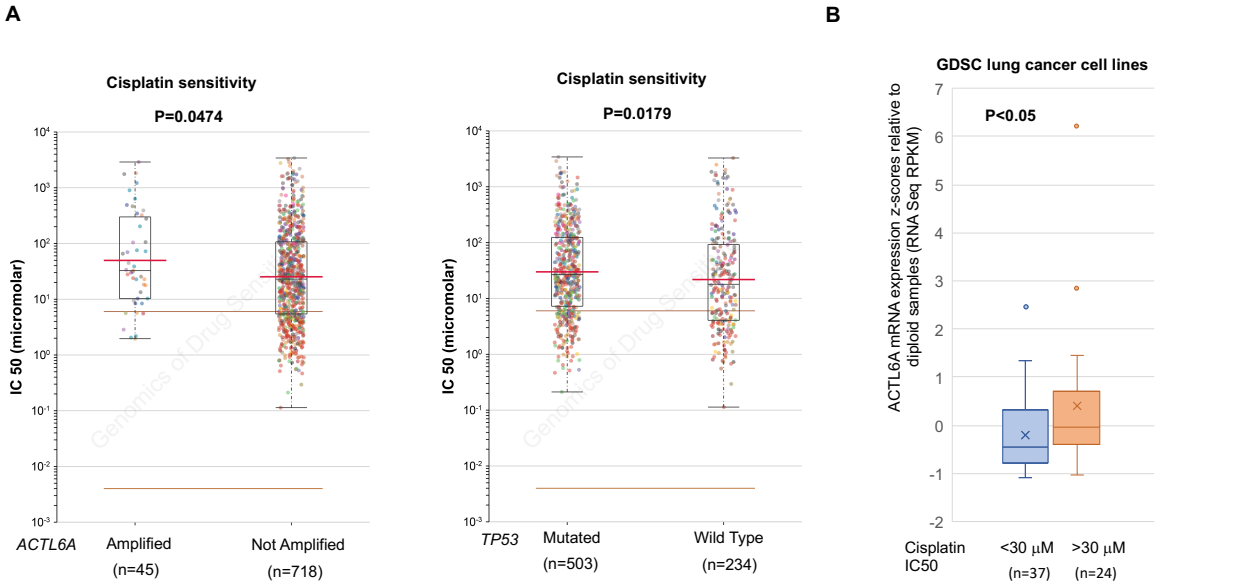
H1299 cells (10^6/sample) were injected subcutaneously into both flanks of 6-8 week-old NOD *scid* IL2 receptor γ chain knockout (NSG) mice. When the tumor volume reached around 50-100 mm^3, cisplatin (5 mg/kg) and/or panobinostat (10 mg/kg) were given intraperitoneally twice per week

for 2-3 weeks. Appropriate vehicle controls (PBS for cisplatin and DMSO for panobinostat) were given to the mice not receiving cisplatin or panobinostat in the combination study. The tumor volume was measured by the caliper and calculated based on the formula $\pi/6$ (length x depth x width). The evaluator was blinded to the group allocation during monitoring. Mice were sacrificed three to four days after the final treatment. Tumors were harvested, weighted and processed for further analysis. The final tumor volume and tumor weight of the xenografts on each mouse were calculated by averaging both sides of tumors. All the procedures of animal work were performed under the Institutional Animal Care and Use Committee (IACUC)-approved protocol and all experiments confirmed to IACUC standards and ethical regulations.

Statistical analysis

The *t* test (two tailed) was used to compare the differences between two experimental groups. *P* values of less than 0.05 were considered statistically significant. Kaplan-Meier curves in lung cancer patients and ovarian cancer patients with the high or low expression of ACTL6A were generated using KM Plotter server, kmplot.com. Patients were split to either three tertiles or four quartiles according to ACTL6A expression. The Kaplan-Meier curves of lower tertile (T1) vs. upper tertile (T3) or lower quartile (Q1) vs. upper quartile (Q4) were compared. Datasets GSE68465, GSE26712 and GSE26193 were extracted from NCBI GEOR2. GDSC dataset was analyzed/downloaded from cancerrxgene.org.

Suppl. Figure S1

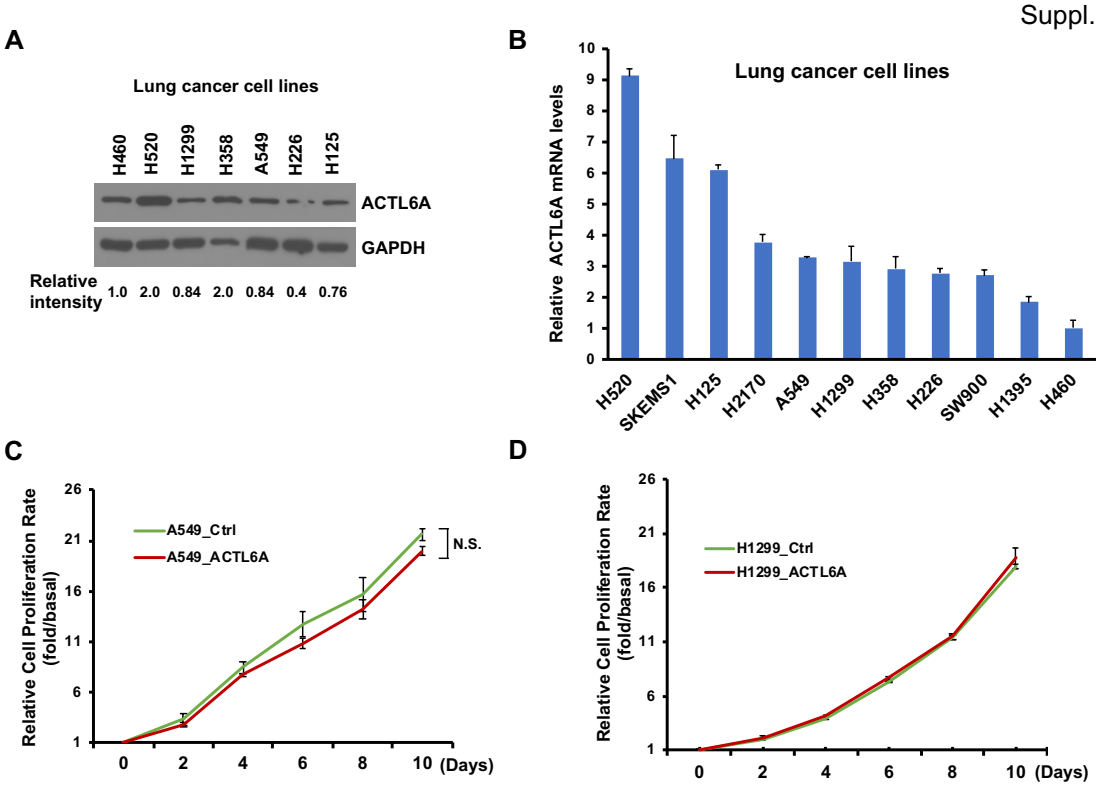


Suppl. Figure S1. *ACTL6A* gene amplification or overexpression is associated with cisplatin resistance in cancer cell lines.

A, Cancer cell lines in GDSC (Genomics of Drug Sensitivity in Cancer) dataset were categorized into two groups according to the presence or absence of *ACTL6A* gene amplification (featured as cnaPANCAN246, and also known as cnaLUSC26 in squamous cell carcinoma of the lung) (left panel) or *TP53* mutations (right panel). The IC_{50} of cisplatin for each cell line was then analyzed.

B, GDSC lung cancer cell lines were categorized into two groups according to cisplatin IC_{50}. The expression of ACTL6A in each cell line was then analyzed.

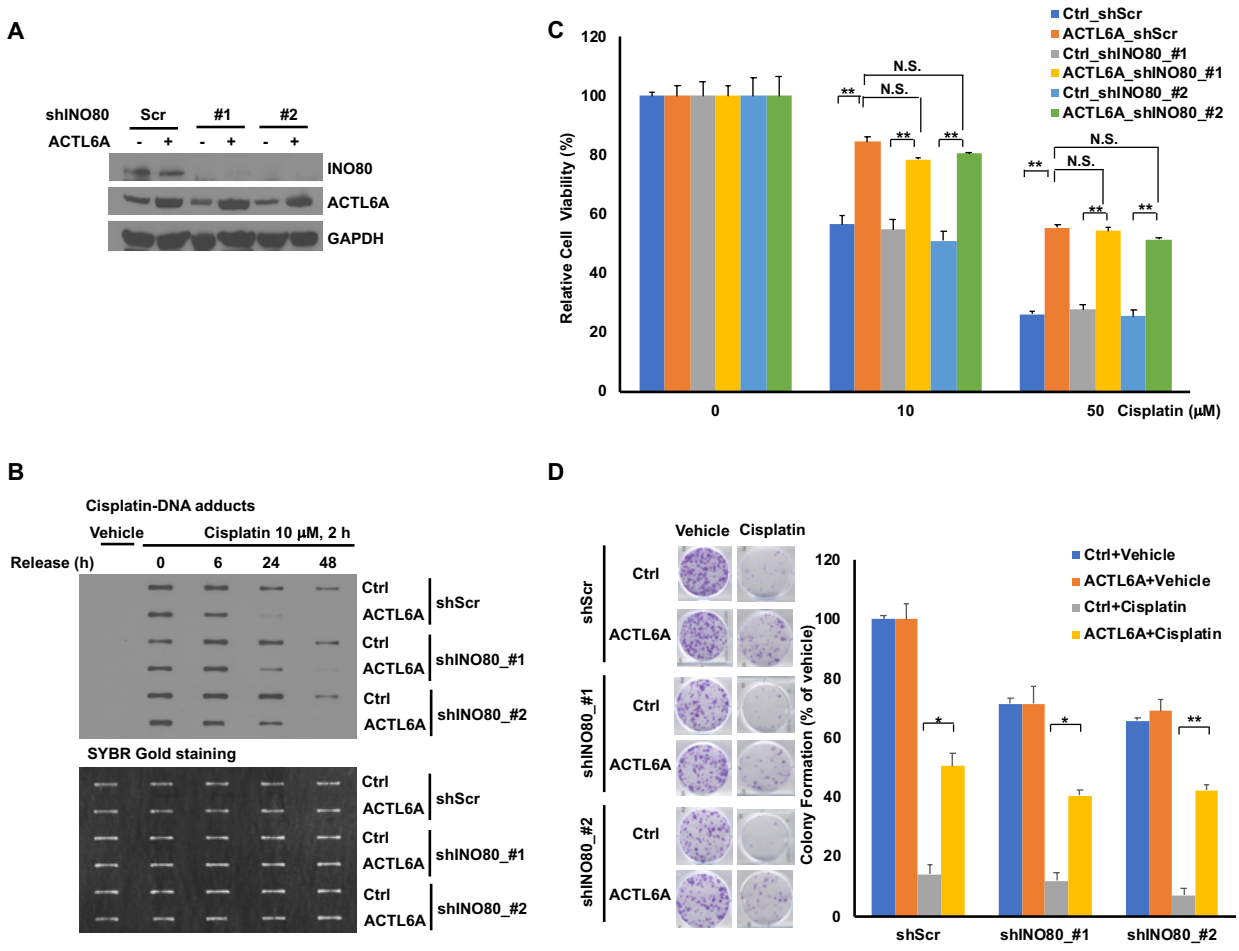
Suppl. Figure S2



Suppl. Figure S2. ACTL6A expression in different lung cancer cell lines.

A, Western blot analysis of ACTL6A protein in a panel of lung cancer cell lines. The intensity of ACTL6A or GAPDH was quantified using ImageJ software. The relative intensity of ACTL6A was normalized to GAPDH in each cell line, and then the fold change was calculated by comparing with that in H460 cells. **B**, Relative ACTL6A mRNA expression in different lung cancer cell lines. ACTL6A mRNA levels in each cell line were determined by quantitative real-time PCR, and were compared with that expressed in H460 cells. **C**-**D**, Overexpression of ACTL6A does not alter the cell proliferation rate in A549 (C) or H1299 (D) lung cancer cells. Equal amounts of cells harboring an empty vector (Ctrl) or ACTL6A were seeded and cultured for various times, followed by the MTT cell proliferation assay. Data shown represent the mean±SD from at least three or four biological replicates. N.S., not significant.

Suppl. Figure S3

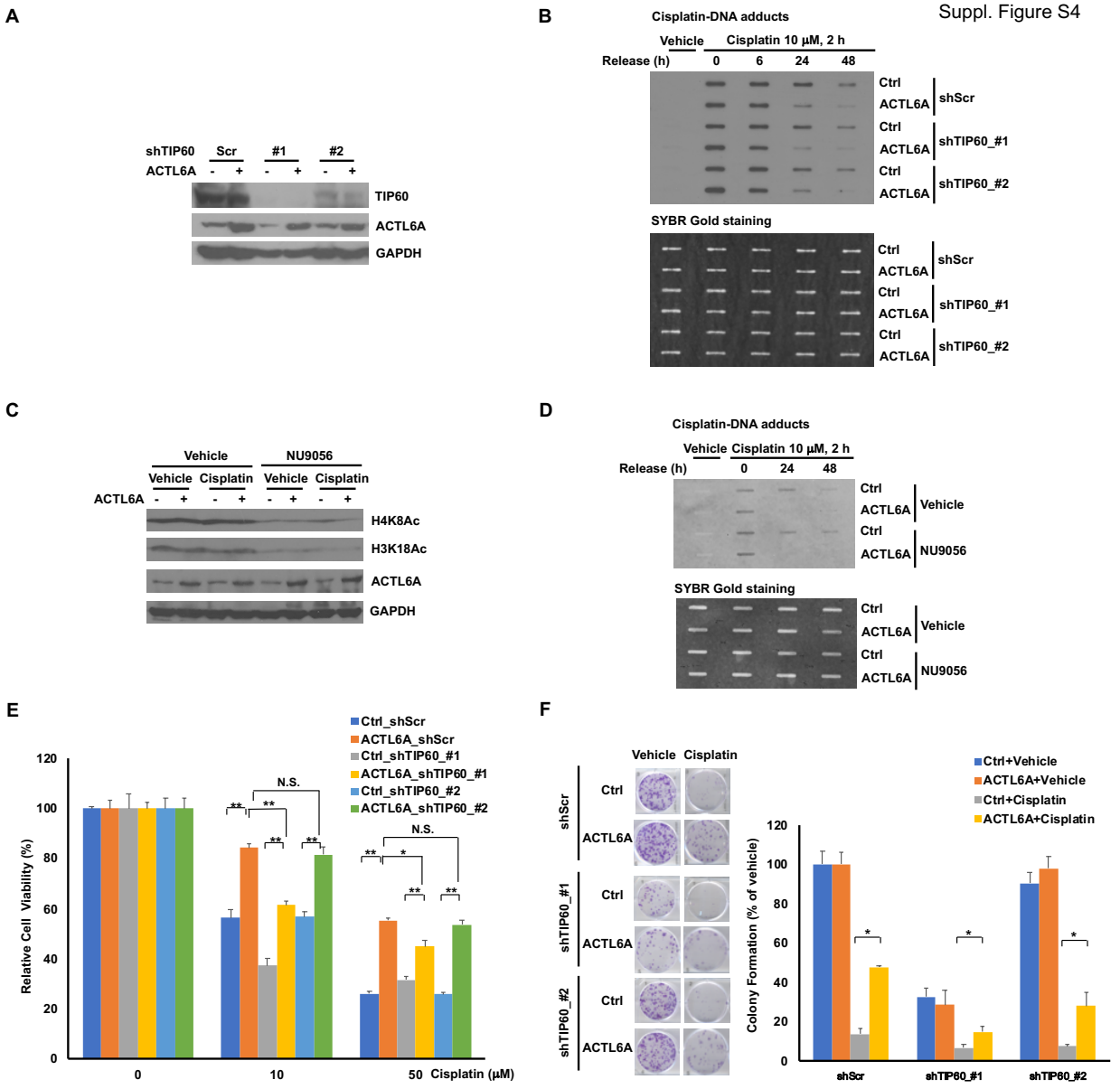


Suppl. Figure S3. The effect of ACTL6A on enhancing cisplatin resistance is not significantly affected by depletion of INO80 complex.

A-B, Depletion of INO80 barely or slightly impairs ACTL6A-enhanced DNA repair after cisplatin treatment. H1299 cells stably harboring an empty vector or ACTL6A were infected with a lentivirus expressing either a scrambled shRNA or an INO80 shRNA (shINO80_#1 or #2). Western blot analysis was performed to detect INO80, ACTL6A or GAPDH (A). Cells were treated with 10 μM cisplatin for 2 h, and then were released and cultured in fresh medium for various times as indicated. Genomic DNA was isolated and subjected to slot blot analysis to determine the DNA repair (B). **C-D,** Depletion of INO80 does not affect ACTL6A-mediated cisplatin resistance in cell viability and clonogenic survival assays. Transfected H1299 cells as

described in (A) were treated with cisplatin, followed by MTT cell viability assay (C). Data shown represent the mean±SD from at least three or four biological replicates. Alternatively, cells were treated with cisplatin, followed by clonogenic cell survival assay (D). Data shown represent the mean±SD from at least three independent experiments. *$p<0.05$ and **$p<0.01$. N.S., not significant.

Suppl. Figure S4



Suppl. Figure S4. TIP60 does not regulate ACTL6A-enhanced cisplatin resistance.

A-B, Depletion of TIP60 has no effect on ACTL6A-enhanced DNA repair after cisplatin treatment. H1299 cells stably harboring an empty vector or ACTL6A were infected with a lentivirus expressing a scrambled shRNA or a TIP60 shRNA (shTIP60_#1 or #2). Western blot analysis was performed to confirm the knockdown effect of TIP60 (A). Cells were treated with cisplatin for 2 h. After release and culture in fresh medium for various times, slot blot analysis was performed (B) as described in Fig. 4B. **C-D,** Inhibition of TIP60 activity with a chemical

inhibitor NU9056 does not affect ACTL6A-enhanced DNA repair after cisplatin treatment. H1299 cells stably harboring an empty vector or ACTL6A were treated with vehicle or 10 μM cisplatin in the absence or presence of 10 μM NU9056 (Santa Cruz Biotechnology) for 24 h, followed by Western blot analysis using an antibody specific to acetyl Histone H4K8 or acetyl Histone H3K18 to confirm the inhibitory activity of NU9056 (C). Alternatively, cells were pretreated with vehicle or 10 μM NU9056 for 16 h, followed by cisplatin treatment for 2 h. After release and culture in fresh medium for various times, slot blot analysis was performed (D) as described in Fig. 4B. **E-F,** Depletion of TIP60 does not affect ACTL6A-mediated cisplatin resistance in cell viability and clonogenic survival assays. H1299 stable cells as described in (A) were treated with cisplatin, followed by MTT cell viability assay (E). Data shown represent the mean±SD from at least three or four biological replicates. Alternatively, cells were treated with cisplatin, followed by clonogenic cell survival assay (F). Data shown represent the mean±SD from at least three independent experiments. *$p<0.05$ and **$p<0.01$. N.S., not significant.

SI References:

1. Khavari PA, Peterson CL, Tamkun JW, Mendel DB, & Crabtree GR (1993) BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. *Nature* 366(6451):170-174.
2. Muchardt C, Bourachot B, Reyes JC, & Yaniv M (1998) ras transformation is associated with decreased expression of the brm/SNF2alpha ATPase from the mammalian SWI-SNF complex. *EMBO J* 17(1):223-231.
3. Lu W*, et al.* (2015) Actl6a protects embryonic stem cells from differentiating into primitive endoderm. *Stem Cells* 33(6):1782-1793.
4. Sarbassov DD, Guertin DA, Ali SM, & Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307(5712):1098-1101.
5. Behrens BC*, et al.* (1987) Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res* 47(2):414-418.
6. Masuda H*, et al.* (1988) Increased DNA repair as a mechanism of acquired resistance to cis-diamminedichloroplatinum (II) in human ovarian cancer cell lines. *Cancer Res* 48(20):5713-5716.
7. Ho SR, Lee YJ, & Lin WC (2015) Regulation of RNF144A E3 Ubiquitin Ligase Activity by Self-association through Its Transmembrane Domain. *J Biol Chem* 290(38):23026-23038.
8. Ho SR, Mahanic CS, Lee YJ, & Lin WC (2014) RNF144A, an E3 ubiquitin ligase for DNA-PKcs, promotes apoptosis during DNA damage. *Proc Natl Acad Sci U S A* 111(26):E2646-2655.