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Ku80 negatively regulates the expression of OCT4 via competitive binding to SALL4 and promoting lysosomal degradation of OCT4

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Highlights

- Ku80 physically interacted with SALL4

- Ku80-SALL4 interaction competitively disrupts the SALL4-OCT4 complex and result in OCT4 lysosomal degradation
- Ku80 inhibits self-renewal and metastasis of HCC through breaking the SALL4-OCT4 interactions and down-regulating the expression of OCT4.

Abstract

SALL4 and OCT4, along with other pluripotency-associated transcription factors, play critical roles in maintaining embryonic stem cell pluripotency and self-renewal. Ku80 is a component of the protein complex called DNA-dependent protein kinase, which mainly involved in DNA double-strand break repair. In this study, we show evidence that Ku80 physically interacted with SALL4. The interaction competitively disrupts the SALL4-OCT4 complex and result in OCT4 lysosomal degradation. Finally, Ku80 inhibits self-renewal and metastasis of hepatocellular carcinoma cells through breaking the SALL4-OCT4 interactions and down-regulating the expression of OCT4. Our study reveal novel function of Ku80 in stemness maintaining of cancer stem cells via its interaction with SALL4 and highlight the double-sidedness of Ku80 as an anti-cancer target.

Abbreviations: ESC, Embryonic stem cell; CSC, Cancer stem cell; DSBs, double-strand breaks; HCC, hepatocellular carcinoma; CO-IP, co-immunoprecipitation; MS, Mass spectrometry; LC-MS, liquid chromatography tandem mass spectrometry; FASP, filter aided sample preparation; DTT, dithiothreitol; IAA, iodoacetamide; TFA, trifluoroacetic acid.

Key words: SALL4, OCT4, Ku80, lysosomal degradation, stemness

1. Introduction

SALL4 (sal-like 4) is a member of the mammal homologs of Drosophila homeotic gene spalt (sal), which is an essential factor for the maintenance of self-renewal of embryonic and hematopoietic stem cells (Yang et al., 2008). SALL4 forms a core transcriptional network with OCT4, Nanog, and Sox2 to drive embryonic stem cell (ESC) and cancer stem cell (CSC) self-renewal (Tatetsu et al., 2016; Yang et al., 2008). SALL4 was recently found to directly connect with the epigenetic modulator NuRD complex (Lu et al., 2009), thus to silence tumor-suppressor genes, such as PTEN. A pharmacologic peptide which disrupts the SALL4–NuRD complex resulted in unidirectional up-regulation of transcripts, turning SALL4 from a dual transcription repressor-activator mode to singular transcription activator mode, and displays significant antitumor effects (Liu, B.H. et al., 2018). Therefore, the study of SALL4 and its interacting proteins may offer an intriguing potential therapeutic target for cancer.

OCT4, a member of the POU family, exerts a fundamental role in the maintenance of naïve pluripotency as a master transcription factor. OCT4 plays an important role not only during embryogenesis but also in tumorigenesis. OCT4 is highly expressed in a variety of cancers and is associated with poor outcomes (Villodre et al., 2016), down-regulation of Oct4 may reduce stemness, proliferation, migration, invasion, chemoresistance, and tumorigenesis of cancer (Lu et al., 2013). Previous studies have shown that the SALL4 interacting and forming a regulatory circuit with Oct4, Nanog and Sox2, and the transcriptional factor complex

is essential for the maintenance of stemness of ES cells (Tanimura et al., 2013; Yang et al., 2008; Yang et al., 2010). Therefore, the search of novel proteins that regulate the interaction of these core transcription factor complexes may provide potential targets for the treatment of cancer targeting cancer stem cell.

Ku80: product of the XRCC5 (X-Ray Repair Cross Complementing 5) gene, is well known for its critical role in the repair of double-strand breaks (DSBs) and was recognized as a target to increase the chemo-/radio-sensitization of tumor cells (Fell and Schild-Poulter, 2015). Our previous proteomic analysis of the side populations of different hepatocellular carcinoma (HCC) cell lines indicates that Ku80 may participate in the metastatic potential of liver CSCs (Liu, H. et al., 2018).

The present study demonstrates that SALL4 physically interacted with Ku80, and the interaction competitively disrupts the SALL4-OCT4 complex then result in OCT4 lysosomal degradation. Finally, Ku80 inhibits the self-renewal and metastasis features of hepatocellular carcinoma cells via breaking the SALL4-OCT4 interactions and down-regulating the expression of OCT4. Our study provides further proof-of principle that SALL4-Ku80 complex as a drug target for treatment of HCC.

2. MATERIALS AND METHODS

2.1. Cell lines and transfection

HEK293T, HepG2, Huh7 and SK-Hep-1cell lines were purchased from ATCC. BEL-7401 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Transfection was performed using lipofectamine 3000 (Invitrogen) according to

the manufacturer's instructions.

2.2. Immunoprecipitation

All immunoprecipitation procedures were carried out at 4°C. Cells were harvested and washed twice with PBS and then lysed in lysis buffer (50 mM HEPES(pH 7.4)，100 mM NaCl，10% glycerol，1% Triton-X-100, 1.5 mM $MgCl_2$, 25 mM NaF, 1 mM PMSF) supplemented with 1X Complete Protease Inhibitor Cocktail (Roche). The lysates were then incubated with the appropriate antibody: anti-SALL4 (Abnova, 1:250), anti-Flag (Abmart, 1:250)，anti-GFP (Abways, 1:250), (mormal IgG was used as negative control) and Dynabeads Protein G (Invitrogen). The mixture was then slowly shaked rotating shaker at 4°C for 3 h. The immuno-precipitate was collected, washed three times with lysis buffer. The samples were boiled with 2× SDS loading buffer for 5 min, and the amount of specific proteins was measured by Western blotting.

2.3. Mass spectrometry

MS of SALL4 interaction protein in 293T cells was performed as previously described (Xing et al., 2019). In brief, cell was cultured in 10cm dishes and then transfected with Myc-SALL4 or not and cultured for another 24 hours. Cells were harvested and the immuno-precipitate was collected as described above using anti-Myc antibody (Origene, 1:250), and the proteins were eluted from the beads with elution buffer (0.1 M Glycine, pH 3.5). The protein concentration was measured using the BCA Protein Assay Kit (Transgen Biotech). Each 6 mg protein mixture was reduced by dithiothreitol (DTT) with a final concentration 8 mM for 30 min at 55 °C. And the following free cysteines were alkylated by 50 mM iodoacetamide (IAA) in the darkness at room temperature for 30 min. The proteins were then purified via the filter aided sample preparation (FASP) protocol with spin ultrafiltration units of molecular weight cut off 10 000 Dalton, and then digested with trypsin (1:100, w/w).

Finally, the digestion was stopped using 4% trifluoroacetic acid (TFA) and the peptides were dried followed by LC-MS/MS analysis using the EASY-nLC1000 system (Thermo Fisher Scientific, Bremen, Germany) and the Thermo Fisher Scientific Q-Exactive Plus quadrupole-Orbitrap mass spectrometer.

2.4. Western blot

Cells were harvested and lysed in lysis buffer (50 mM HEPES(pH 7.4), 100 mM NaCl, 10% glycerol, 1% Triton-X-100, 1.5 mM $MgCl_2$, 25 mM NaF, 1 mM PMSF) supplemented with 1X Complete Protease Inhibitor Cocktail (Roche). Protein was separated by SDS-PAGE and electrically transferred to a nitrocellulose membrane. The membrane was probed with the appropriated primary antibody: anti-Myc (Origen, 1:1000), anti-Flag (Abmart, 1:1000), anti-SALL4 (Abnova, 1:1000), anti-Ku80 (Invitrogen, 1:1000), anti-GFP (Abways, 1:1000), anti-Nanog (Invitrogen, 1:1000), anti-Oct4 (Abcam, 1:1000), anti-Sox2 (CST, 1:1000), anti-Actin (Abcam, 1:1000) and with an HRP-conjugated secondary antibody. Blots were visualized by ECL.

2.5. Immunofluorescent staining and microscopic observation

Cells were transfected with expression vector as required (GFP-Ku80 & mutants，Flag-SALL4 & mutants，Myc-Oct4，Oct4-GST-GFP). After 24h, cells were fixed in 4% paraformaldehyde. Cells were incubated with anti-Flag (Abways, 1:200), anti-Myc (Origene, 1:200), anti-Ku80 (Invitrogen, 1:200) and anti-SALL4 (Abnova, 1:200) antibody, followed by Alexa flour 546-conjugate secondary antibody (Thermo Fisher Scientific，1:200). Cell were then stained with 4’,6-diamidino-2-phenylindole (DAPI) to indicate the nuclei and visualized using a confocal microscope (Zeiss LSM 780).

2.6. Tumor Spheroid formation assay

Cells were plated in ultralow attachment 12-well pates (Corning, USA) at a

density of 1×10^4 cells/ml in serum-free DMEM/F12 medium (Invitrogen, USA) supplemented with 20 ng/ml epidermal growth factor (Sigma, USA), 10 ng/ml basic fibroblast growth factor (Sigma), 5 mg/ml insulin (Sigma), 1×B27 supplement (Invitrogen) and 0.4% bovine serum albumin (Sigma). Cells were cultured under 5% CO_2 at 37°C for a week. The spheres were photographed using a phase contrast microscope.

2.7. Trans-well invasion, migration and wound healing assays

Cell migration was assayed using the trans-well method, with 8-μm pore filters (Corning, NY). The lower chamber was filled with DMEM, supplemented with 10% FBS, and 2×10^4 cells in 0.5 ml of DMEM were loaded into the upper chamber. After a 20-hour incubation, the cells that migrated to the bottom of the membrane were fixed with 4% formaldehyde. The cells on the top of the membrane were removed by wiping the surface with a cotton swab. The cells were stained with 0.5% crystal violet and observed under a microscope. The number of migrated cells was counted at a magnification of 200× from five adjacent microscope fields. For the Matrigel invasion assay, the procedures used were the same as those described above, except that the trans-well membrane was coated with Matrigel (BD Biosciences) to form a matrix barrier. Wound healing assay was carried out using culture inserts (IBIDI, Germany). Cells were seeded into culture inserts in a 6 well plate and were incubated to allow them to adhere. The culture insert provided two cell culture reservoirs that were separated by approximately a 0.5mm thick wall. The culture insert was removed after the cells were cultured to full confluence, a ‘wound’ of approximately 0.5mm was formed between the two cell patches. Wound closure was observed after 24 h and was photographed under a microscope. The fraction of cell coverage across the line represents the migration rate.

2.8. Statistical analysis

The data were analyzed using GraphPad Prism, version 5 (GraphPad Software). The results are expressed as the mean ± SEM. Statistical analyses were performed using the Student's t-test. $p < 0.05$ was considered statistically significant.

3. Result

3.1 Ku80 physically interacts with SALL4

To identify novel SALL4-binding proteins, 293T cells were transfected with Myc-SALL4 vector. Endogenous proteins interacting with SALL4 were then immuno-precipitated using anti-myc antibody. The immuno-precipitate was analyzed with high-resolution liquid chromatography tandem mass spectrometry (LC-MS/MS) to identify the proteins associated with SALL4 (Figure. 1A). One of the SALL4-associated proteins identified by MS was Ku80 (Figure. 1A). We next explore whether SALL4 and Ku80 colocalized with each other in cells. Immunofluorescence staining show both exogenous and endogenous Ku80 and SALL4 colocalized in the nucleus of 293T and HepG2 cells respectively (figure. 1B). The SALL4-Ku80 interaction was confirmed by co-immunoprecipitation both in exogenous level and endogenous level (Figure. 1C). Thus, we identify a novel SALL4 interacted protein, Ku80.

To further determine which domain of Ku80 binds to SALL4, different Ku80 truncation mutants were used (Figure. 1D). CO-IP assay shows that, Flag-SALL4 efficiently pulled down full-length Ku80 and Ku80/C1 (449-732), but not Ku80/N1 (1-334) or Ku80/N2 (1-449) (Figure.1D), indicating that C terminal region of Ku80 (amino acid 449-732) is required for SALL4 binding. The subcellular localization of these truncation mutants confirmed that Ku80/C1 but not Ku80/N1 and Ku80/N2 colocalized with SALL4 (Figure S1). We then mapped the SALL4 region that interacts with Ku80. The CO-IP assay using different truncated SALL4 mutants shows that Ku80 interacted with N1,

N2 and C2, but not with C1 (Figure. 1E), indicating that the amino acid 300-500 region was critical for the interaction. The subcellular localization of these truncation mutants was also examined by immunofluorescence staining as shown in Supplementary Figure S2.

3.2. Ku80 induced OCT4 lysosomal degradation

SALL4, OCT4, Sox2 and Nanog, which formed an interconnected autoregulatory circuit, wherein each of the four factors can regulate its own expression as well as that of others (Lim et al., 2008; Tatetsu et al., 2016). Thus we wonder if binding of Ku80 to SALL4 may affect the expression of SALL4 and other stemness-related transcriptional factor. As shown in supplementary figure S3, over-expression of Ku80 did not affect the mRNA level of SALL4 or other stemness-related transcriptional factor. Similarly, the protein level of SALL4, NANOG and SOX2 keep unchanged upon Ku80 over-expression, while interestingly, Ku80 significantly down-regulated the protein level of OCT4 (Figure 2A). To further confirm this result, different dose of Ku80 expression vector was transfected into HepG2 and 293T cells in the absence or presence of OCT4 expression vector, and the data clearly indicated that Ku80 significantly reduced the protein expression of OCT4 in a dose-dependent manner (Figure 2B). To further confirm that Ku80 negatively regulates OCT4 expression, the expression of endogenous Ku80 was knockdown by siRNA in BEL-7401 cells. As expected, the expression of OCT4 was increased (Figure 2B, right panel).

Since that Ku80 significantly inhibited the protein level of OCT4 without affect its mRNA level, we suspected that Ku80 down-regulated the expression of OCT4 at the post-transcriptional level. Ubiquitin-proteasome pathway and lysosome pathway are the two most important pathways for protein degradation. To determine which pathway is involved in Ku80- promoted OCT4 degradation, Ku80 was transfected alone or co-transfected with OCT4 in HepG2 cells and 293T cells and then treated with proteasome inhibitor MG132

and lysosome inhibitor NH_4Cl. As shown in figure 2C, MG132 treatment does not affect the degradation of OCT4 induced by Ku80. However, NH_4Cl treatment strongly block Ku80 promoted OCT4 degradation. Thus this data indicated that Ku80 down-regulated the protein level of OCT4 by promote its lysosomal degradation. Another proteasome inhibitor epoxomicin and autophagy inhibitor chloroquine were used to further confirm our finding. As expected, chloroquine but not epoxomicin reverse down regulation of OCT4 at the presence of Ku80 (Figure 2C).

Since lysosomes accumulated in the cytoplasm and OCT4 mainly located in the nuclear, nuclear-cytoplasmic translocation of OCT4 was needed before its lysosomal degradation. Then, immuno-fluorescence staining assay confirmed that GFP-Ku80 did induced the nuclear-cytoplasmic translocation of OCT4 (Figure 2D). To further confirm that cytoplasmic translocation of OCT4 induced by Ku80 is a necessary pre-condition for its degradation, a fusion expression vector of OCT4 (OCT4-GST-GFP) was used. Pervious study has show that OCT4 is a nucleocytoplasmic shuttling protein and is primarily exported via passive diffusion(Oka et al., 2013). The fusion expression vector OCT4-GST-GFP with a molecular mass of more than 90 kDa, is large enough to prevent its passive diffusion(Oka et al., 2013). Subcellular localization of OCT4-GST-GFP was examined in the presence or absence of Ku80. As expected, Ku80 was unable to induced export of OCT4-GST-GFP (Figure 2E), and more importantly, the protein level of OCT4-GST-GFP kept un-changed when co-transfected with different dose of Ku80 (Figure 2F). Thus, the above data confirmed that Ku80 down-regulated the expression of OCT4 by inducing its export and degradation in lysosome.

3.3. Ku80 inhibit OCT4 via SALL4

Since we first discovered the interaction between ku80 and SALL4 in this study, the function of SALL4 in the process of Ku80 promoted OCT4 degradation was worth for further investigation. Previous studies have

confirmed that SALL4 form a protein complex with OCT4 (van den Berg et al., 2010; Yang et al., 2008). We speculate whether Ku80 competitively binds to SALL4, thereby lead to OCT4 to separate from the SALL4 complex, and then exporting and degradation of OCT4 .

To verify our hypothesis, we transfected Flag-SALL4, Myc-Ku80 and Myc-OCT4 expression vectors into 293T cells. Simultaneous detection of Ku80 and OCT4 in Flag-SALL4 immuno-precipitates by using anti-Myc antibody was performed. As shown in figure 3A, we detected a strong interaction between OCT4 and SALL4 when co-transfected the Flag-SALL4 and Myc-OCT4 into 293T cells. As we expected, by further co-transfection with Myc-Ku80 in 293T cells, we detected the binding between SALL4 and ku80. Simultaneously, the interaction of SALL4 and OCT4 was significantly reduced. Therefore, this result indicated that Ku80 inhibited the interaction of SALL4 and OCT4 by competitively binding to SALL4, which may sequester SALL4 from OCT4 and thus OCT4 will release and undergoing its degradation.

To further confirm that Ku80 competitively binds to SALL4 and leads to degradation of OCT4, different Ku80 truncation mutants were used to examine Myc-OCT4 expression in 293T cells. The protein levels of Myc-OCT4 in 293T cells were reduced when transfected with Ku80 and its truncation mutants that interacted with SALL4 (Ku80/C1) (Figure 3B). In contrast, we did not observe the reduction of OCT4 expression in cells transfected with Ku80/N1 and Ku80/N2, which did not interacted with SALL4 (Figure 3B). Clearly, these results demonstrate that interaction with SALL4 is a prerequisite for ku80 to inhibit OCT4 expression. Meanwhile, the subcellular localization of OCT4 was detected by confocal microscope under co-transfection with the Ku80 truncation mutants. As expected, Ku80/C1 induced translocation of OCT4 from nuclear to cytoplasm. Conversely, Ku80/N1 and Ku80/N2 was unable to induce cytoplasmic translocation of OCT4 (Figure 3C). Taken together, these results clearly demonstrated that Ku80-SALL4 interaction led to the separation of OCT4 from SALL4 and thus OCT4 was released and translocated to

cytoplasm and finally underwent its lysosomal degradation.

3.4. Ku80 suppresses self-renewal and metastasis of HCC.

It is well documented that SALL4 and OCT4 are both as CSCs markers, participated in regulating self-renewal and metastasis of cancer cells (Tatetsu et al., 2016; Zeineddine et al., 2014). We next investigated the biological consequences of Ku80 on self-renewal and metastasis phenotypes of HCC cells. Tumor sphere formation assay show that Ku80 significantly inhibited spheroid formation capacities of HCC cells (Figure 4A). Trans-well assays showed that both the migratory and invasive activities of HCC cells were suppressed by Ku80 (Figure 4B). The wound healing assay further confirmed that Ku80 negatively regulated HCC cells' migration (Figure 4C). Next, different Ku80 truncation mutants were used again to further confirm that the role of Ku80 on regulating self-renewal and metastasis depend on its interaction with SALL4. As shown in figure 4D, Ku80/C1 but not Ku80/N1 or Ku80/N2 remain its ability to suppress HCC cell migration and invasion. Similarly, Ku80/C1 inhibits the tumor sphere formation of HCC cells rather than Ku80/N1 (Figure. 4F).

In summary, these results suggest that Ku80 may repress HCC metastasis and self-renewal via its interaction with SALL4.

4. Discussion

As a DNA-binding subunit of the DNA-PK complex, Ku80 mainly involved in DNA DSB repair (Fell and Schild-Poulter, 2015). However, the functions of Ku80 beyond damage repair are still remaining further investigation. A study in HCC has showed that Ku80 functions as a tumor suppressor by suppressing cell proliferation (Wei et al., 2012), while another study in lung cancer cells indicated that Ku80 promote tumor growth by up-regulating COX-2 expression (Xiao et al., 2015). Prior studies through various experimental models indicate

that Ku80 may act as either a tumor suppressor or an oncoprotein. Here we show that Ku80 inhibited HCC cell self-renewal and metastasis by down-regulating the expression of OCT4. More importantly, different from previous studies on Ku80 function, our study reported for the first time that Ku80 promotes protein degradation through competitively protein interaction.

So far, more and more evidence indicated that Ku80 is involved in regulation of tumorigenesis and tumor progression. However, there is no clearly evidence that Ku80 participated in cancer cell self-renewal regulation. Our previous study using mass spectrometry analyzed the differentially expressed proteins among side population (sp) cells from 4 HCC cell lines with different metastatic potentials, and the data indicated that XRCC5/Ku80 expression decreases in sp cells as the increase of metastatic potentials (Liu, H. et al., 2018), prompt that Ku80 may involved in regulating self-renewal and metastasis, which was consistent with the conclusions of this study.

In our study, over-expression of Ku80 competitively disrupts the interaction between SALL4 and OCT4. Conversely, under normal physiological conditions, CSCs with high level of pluripotency-associated proteins, may possibility disrupt the interaction between SALL4 and Ku80, which make Ku80 prone to play its role in DSB repair. This may provide an explanation for the reason that CSCs are resistance to chemotherapy and radiotherapy.

In summary, our studies identified Ku80 as a novel SALL4 interaction protein. Ku80, via SALL4, inhibited OCT4 protein expression by promoting its lysosome degradation and thus inhibited self-renewal and metastasis of HCC cells. Our study may provide an insight into the underlying mechanisms of the phenomenon that CSCs are resistance to chemo-/ radio-therapy, revealing the double-sided of Ku80 for anticancer strategy.

Authors' contributions

B-X.Z., A-M.H. and X-L.L. designed the project. B-X.Z., X-Y.Z., X-H.T., K.K., F.W., Y-C.W., P.H. and S-B.L. performed the experiments with data analysis; X-H.X, C-L.Z. and Q.L. provided support with experimental techniques. B-X.Z. and X-L.L. wrote the paper.

Competing interests

The authors disclose no potential conflicts of interest.

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Figures & Legends

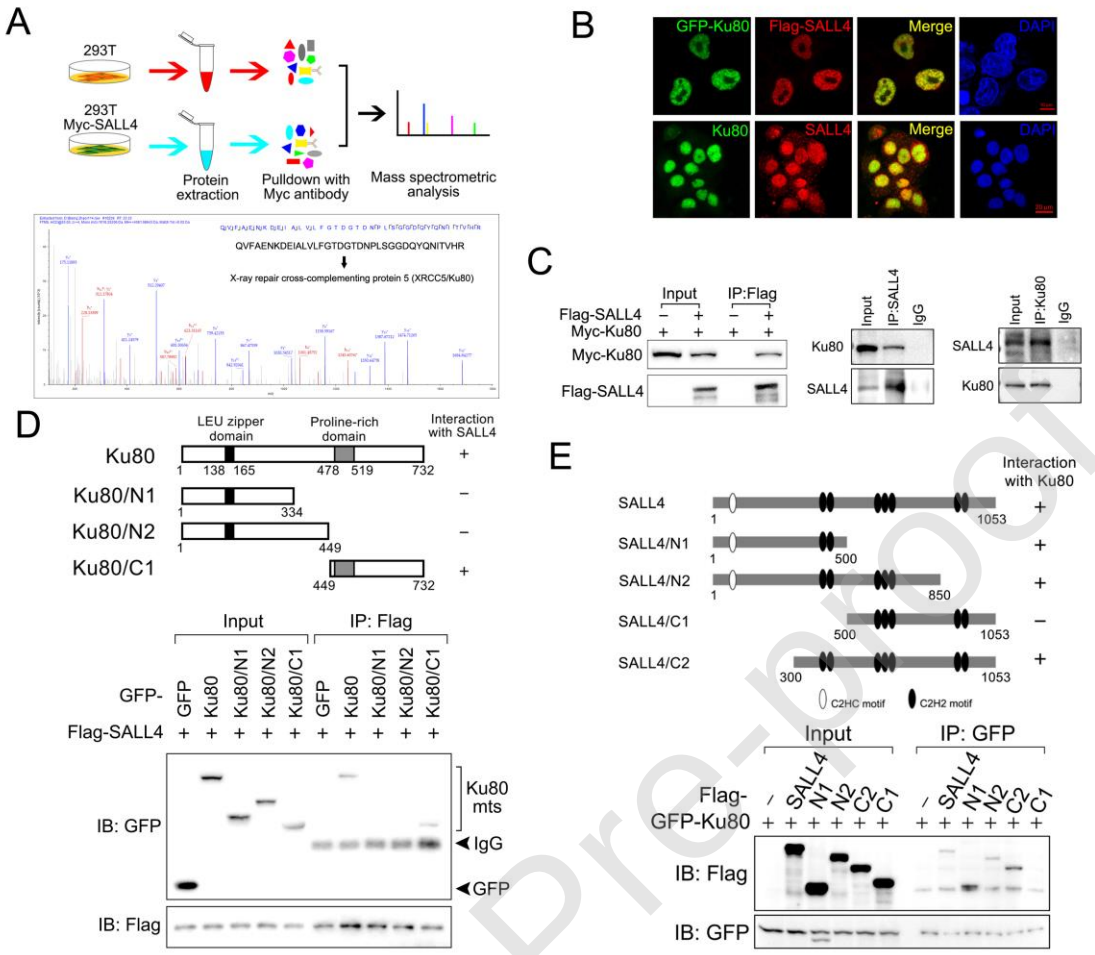

Figure 1. SALL4 physically interacts with Ku80. (A) Schematic overview of mass spectrometric analysis for proteins interacting with SALL4 in 293T cells. Down panel show the peptide mass fingerprint of Ku80. (B) Subcellular localization of exogenous SALL4/Ku80 in 293T cells and endogenous SALL4/Ku80 in HepG2 cells. GFP-Ku80 and Flag-SALL4 were transfected into 293T cells. Cells were immuno-stained for detecting SALL4 by Flag antibody then followed by Alexa flour 546 conjugated secondary antibody. HepG2 cells were immune-stained for anti-SALL4 antibody followed by Alexa flour 546 conjugated secondary antibody and anti-Ku80 antibody followed by Alexa flour 488 conjugated secondary antibody. Cells were visualized with the confocal microscope. (C) Co-IP experiments showed the interaction between SALL4 and Ku80. Flag-SALL4 and Myc-Ku80 were co-transfected into 293T cells, and the cell lysate was immunoprecipitated using anti-Flag antibody. The immune-precipitate was

then examined via western blot using anti-Myc antibody. The input includes 10% of the cell lysate (*left*). Huh7 cell lysate was incubated with SALL4 or Ku80 antibody to immuno-precipitate endogenous SALL4 or Ku80 protein. For western blotting of the immuno-precipitate, Ku80 and SALL4 antibodies was used. IgG was used as a control (*middle & right*). (D) Identification of Ku80 sequence critical for SALL4 binding. Schematic diagrams depict different Ku80 deletion constructs used in the domain mapping experiments (top). 293T cells were transfected with Flag-SALL4 and GFP-Ku80 truncation mutants. Cell lysates were immunoprecipitated with anti-Flag antibody. The immuno-precipitates and cell lysates were then analyzed by western blot using anti-GFP antibody for GFP-Ku80 and its truncation mutants, and anti-Flag antibody for Flag-SALL4. GFP was used as a negative control. (E) Interaction of Ku80 with SALL4 and its deletion mutants. Structures of deletion mutants of SALL4 were shown on the top. 293T cells were transfected with GFP-Ku80 and different Flag-SALL4 deletion mutants as indicated. Cell lysates were immunoprecipitated with anti-GFP antibody. The immuno-precipitates and cell lysates were then analyzed by western blot using anti-Flag and anti-GFP antibody.

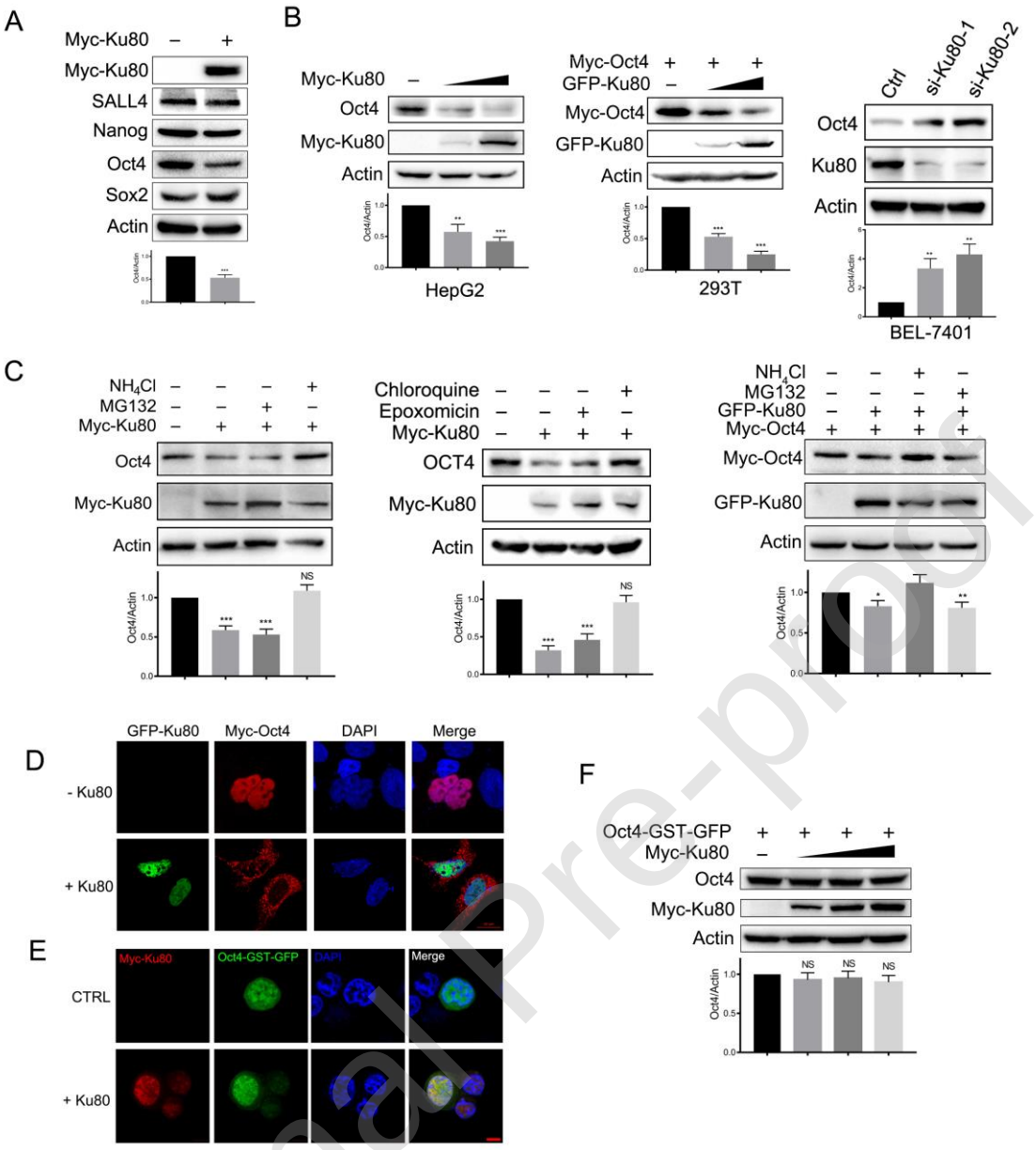

Figure 2. Ku80 induced OCT4 lysosomal degradation. (A) Ku80 down-regulated protein level of OCT4. Myc-Ku80 were transfected into HepG2 cells. The expression of Ku80, SALL4, Nanog, OCT4 and Sox2 were analyzed by western blot. (B) Ku80 inhibited OCT4 expression in a dose-dependent manner. Increasing amounts of Ku80 were transfected in the absence or presence of OCT4 in HepG2 or 293T cells as indicated. BEL-7401 cells were pretransfected with si-Ku80 to repress the expression of endogenous Ku80. Scrambled siRNA was used as a control (si-Ctrl). Cell lysates were prepared and analyzed by western blot using anti-OCT4 or anti-Myc antibody to indicate endogenous and exogenous OCT4. (C) Ku80 induced OCT4 lysosome

degradation. Different expression vectors as indicated were transfected into HepG2 cells (left and middle panel) and 293T cells (right panel) and then treated with MG132 (20 μM, 3h), NH_4Cl (10 mM, 3h) chloroquine (50 μM, 12h) or epoxomicin (1 μM, 24h). The expression of OCT4 and Ku80 were analyzed by western blot. (D) Ku80 triggers OCT4 nuclear export. 293T cells were transfected with GFP-Ku80 and Myc-OCT4 as indicated. Cells were immuno-stained using Myc antibody followed by Alexa fluor 546 conjugated secondary antibody and then visualized with the confocal microscope. (E) Subcellular localization of OCT4-GST-GFP and Ku80 in 293T cells. (F) Ku80 has no effect on expression of OCT4-GST-GFP. Increasing amounts of Ku80 were co-transfected with OCT4-GST-GFP in 293T cells. The expression of OCT4 and Ku80 were examined by western blot. The levels of OCT4 protein were quantified by densitometry. The bars represent the means ± SD values from three independent experiments. * $P < 0.05$, ** $P<0.01$,***$P<0.001$.

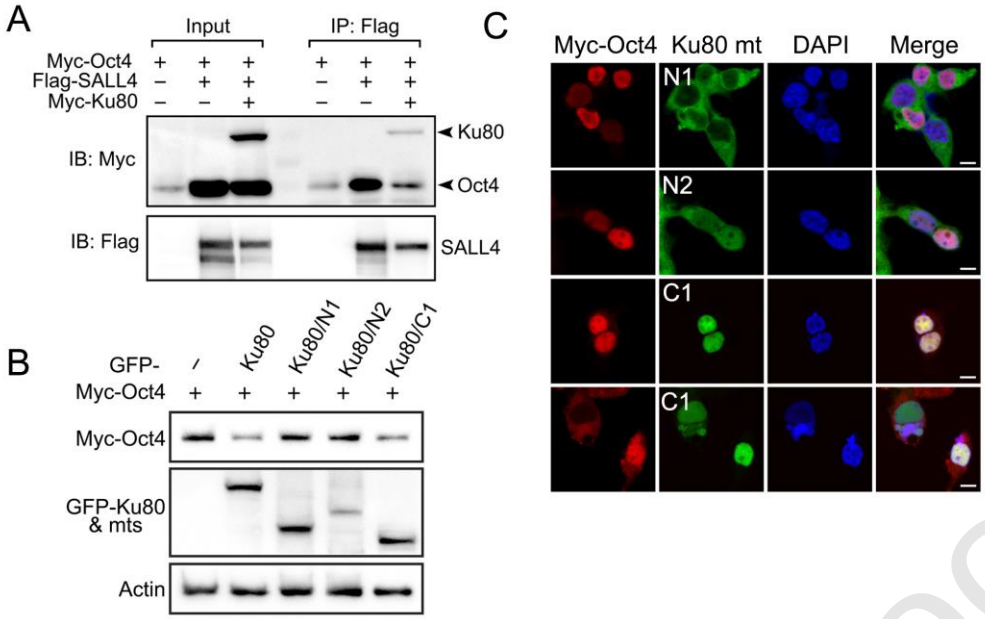

Figure 3. Ku80 inhibits OCT4 via SALL4. (A) Ku80 competitively binds to SALL4 then separates SALL4-OCT4 interaction. Myc-OCT4, Flag-SALL4 and Myc-Ku80 were transfected into 293T cells and the cell lysate was immunoprecipitated using anti-Flag antibody. The immuno-precipitate was then examined *via* western blot using anti-Myc antibody against the common Myc tag of OCT4 and Ku80. (B) Effects of different Ku80 truncation mutants on OCT4 inhibition. GFP-Ku80 and its truncation mutants together with Myc-OCT4 were transfected into 293T cells. The expression of OCT4, Ku80 and its truncation mutants were monitored by western blot. (C) Subcellular localization of Ku80 truncation mutants and OCT4. Myc-Oct4 and Ku80 truncation mutants were transfected into 293T cells and immuno-stained as indicated. Stained cells were visualized under confocal microscope.

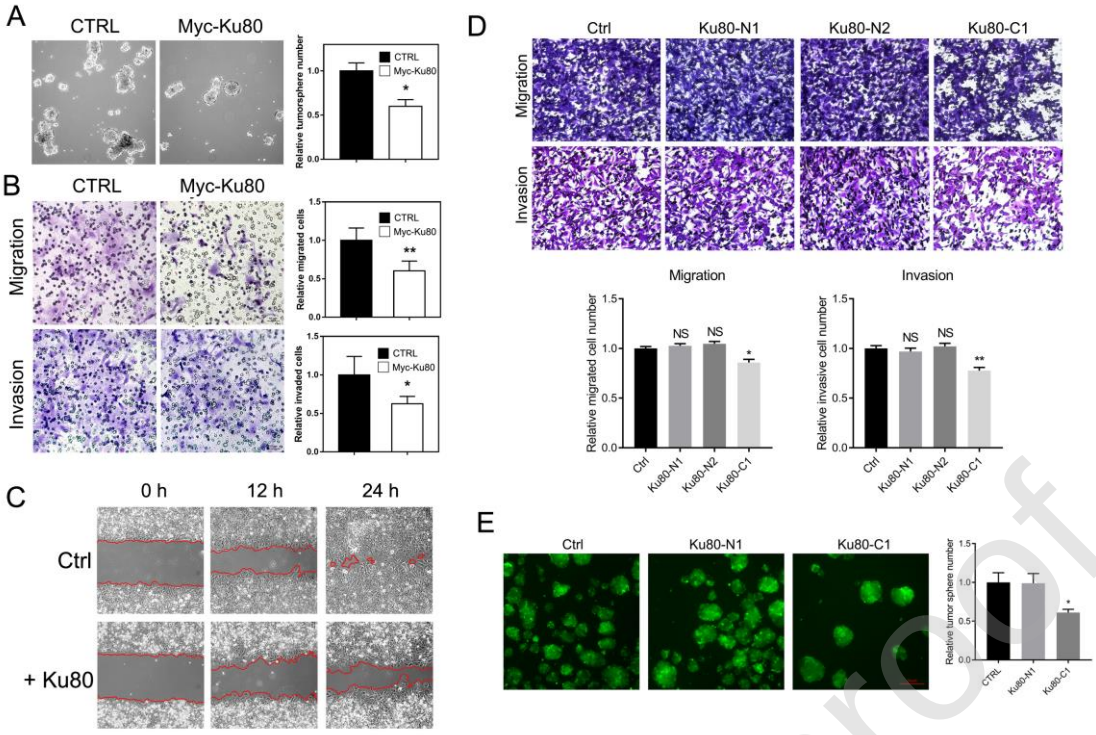

Figure 4. Ku80 suppress self-renewal and metastasis of HCC.

(A) HepG2 cells were transfected with Myc-Ku80 or not, then tumor sphere-forming capacity was analyzed by tumor sphere formation assay. (B) HepG2 cells transfected with Myc-Ku80 or not, then were subjected to trans-well migration and invasion assay. Migrated/invaded cells in fields were quantified and representative photographs were shown. (C) Wound healing assay was performed to determine the cell migratory capacity. (D) SK-Hep-1 cells were transfected with different Ku80 truncation mutants as indicated, and subjected to trans-well migration and invasion assay. (E) HepG2 cells were transfected with different Ku80 truncation mutants as indicated and tumor sphere-forming capacity was analyzed by tumor sphere formation assay.