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ORIGINAL ARTICLE

SALL4 promotes glycolysis and chromatin remodeling via modulating HP1α-Glut1 pathway

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SALL4 has recently been identified to promote chemo-resistance in multiple types of cancer, but the underlying mechanism remains to be fully established. Open chromatin structure is important for DNA damage response (DDR) and DNA repair. Here, we demonstrate that SALL4 promotes open chromatin by destabilizing heterochromatin protein 1α (HP1α) by recruiting ubiquitin E3 ligase CUL4B to HP1α. The silencing of SALL4 in cancer cells decreased the expression levels of Glut1 and inhibited glycolysis in cancer cells. The upregulation of HP1α in human cancer cells suppressed open chromatin, glycolysis and Glut1 expression levels. Therefore, SALL4 promotes the expression of Glut1 and open chromatin through a HP1α-dependent mechanism. Impaired DDR in SALL4-deficient human cancer cells can be rescued by the restored expression of Glut1, indicating the importance of HP1α-Glut1 axis in SALL4-mediated DDR. These findings demonstrate that SALL4 could induce drug resistance by enhancing DDR and DNA repair through promoting glycolysis and subsequent chromatin remodeling.

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INTRODUCTION

Cancer cells and pluripotent stem cells share common characteristics such as glycolytic metabolism.[1,2] The expression of pluripotency factors including Oct4, Sox2 and Nanog is associated with the poor prognosis of cancer patients,[3] and they can promote many aspects of cancer development.[4–6] SALL4 is a zinc finger transcriptional factor that maintains the self-renewal of embryonic stem (ES) cells and embryonic developmental process.[7,8] In adult tissues, SALL4 is only detectable in germ cells and hematopoietic stem cells.[9,10] However, SALL4 is overexpressed in endometrial, gastric, breast, ovarian, and colon cancer, and promotes their tumorigenesis.[11–17] The clinical data indicate a correlation between SALL4 expression with drug resistance and relapse of cancer patients.[17–20]

Recent studies have suggested mechanisms to account for the SALL4-mediated drug resistance. In this context, SALL4 directly regulates the expression of PTEN and c-myc.[13,21] SALL4 could promote drug resistance by increasing the expression of adenosine triphosphate (ATP)-binding cassette (ABC) multidrug transporter family such as ABCB1 and ABCG2.[22] Since many chemotherapeutic drugs are designed to kill cancer cells by inducing DNA damage, the efficiency to repair DNA damage in cancer cells dictates drug resistance. Therefore, the inactivation of DNA repair can sensitize cancer cells to chemo-drugs.[23–25] In support of a direct role of SALL4 in DDR and DNA repair, a recent study has shown that SALL4 promotes Ataxia Telangiectasia Mutated (ATM)–dependent DNA damage response in ES cells by stabilizing the Mre11/Rad50/Nbs1 complex at the site of DNA damage.[26] Here, we demonstrate that SALL4 promotes DDR and DNA repair in cancer cells by promoting glycolysis and open chromatin through destabilizing HP1α.

RESULTS

SALL4 promotes DNA DSB damage responses in cancer cells

Based on a previous report,[18] we chose SNU-398 hepatocarcinoma cell line because this cancer cell line expresses endogenous SALL4 and is drug resistant. The major conclusions were also confirmed in other human cancer cell lines HepG2 and 293 cells. We established a SALL4-knockdown (SALL4 KD) SNU-398 cell line by transducing cells with SALL4 shRNA lenti-virus (Figures 1a and b). Using clonogenic survival assay, we demonstrated that SALL4 KD cells were more sensitive to DNA DSB damage inducing chemotherapy drug doxorubicin (Dox), supporting the notion that SALL4 promotes drug resistance (Figure 1c). In addition, the restoration of the SALL4 protein levels in SALL4 KD cells rescued the sensitivity to the chemotherapy drug, confirming that SALL4 promotes drug resistance (Supplementary Figure 1). Since the activation of ATM is a critical early event of DDR indicated by the autophosphorylation of ATM at Ser1981,[27] we examined the ATM autophosphorylation in SALL4 KD cells and control cells after Dox-induced DNA double-stranded break (DSB) damage, and showed that ATM activation was reduced in SALL4 KD cells when compared to that in control cells (Figures 1d and e). In further support of the finding that the efficiency of DNA repair is reduced in SALL4 KD cells, the levels of DNA DSB damage were significantly higher in SALL4 KD cells than in control cells after Dox treatment (Figure 1f).

SALL4 destabilizes heterochromatin structure protein HP1α

SALL4 was located in DAPI-dense regions in cancer cells, and was co-localized partially with γ-H2AX foci formed at the site of DNA DSB damage (Figure 1e, Supplementary Figures 2a and b). SALL4 was not co-localized significantly with the heterochromatin marker HP1α (co-localization coefficiency, Rr = 0.21) (Figure 2a). Instead, there was an inverse correlation between the protein

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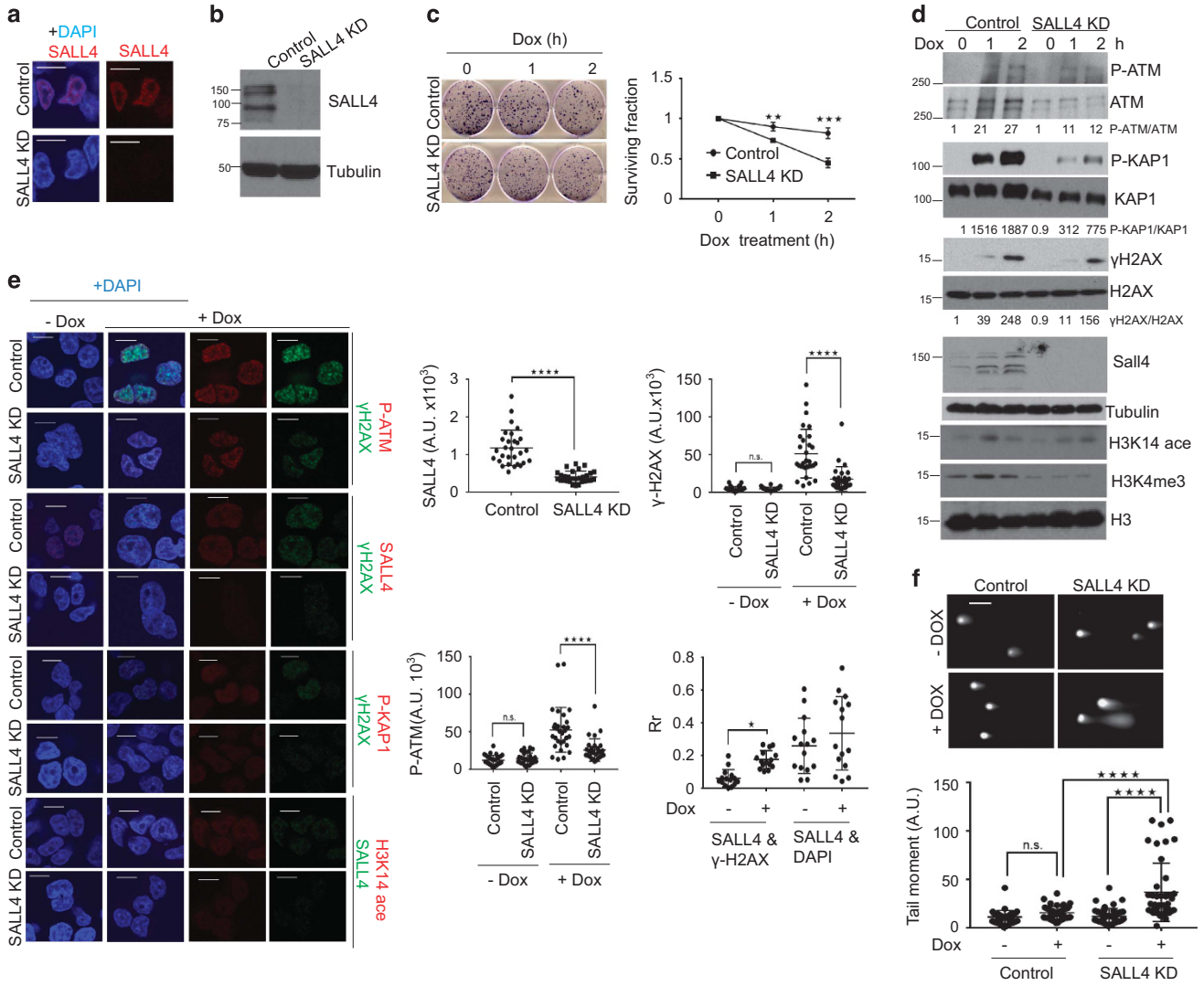


Figure 1. Depletion of SALL4 in human cancer cells impaired DDR and DNA repair, leading to hypersensitivity to Doxorubicin (Dox). The silencing of SALL4 in cancer cells was confirmed by immunofluorescence staining for SALL4 (**a**) and western blotting (**b**). SNU-398 cell lines stably expressing SALL4 shRNA (SALL4 KD cells) and scramble shRNA (control cells) were generated by lentiviral infection and analyzed for SALL4 expression using confocal microscopy. Nuclear DNA is counterstained with DAPI (blue). Representative images for each cell line are shown. Scale bar is 10 μm. (**c**) SALL4 KD cells were hypersensitive to Doxorubicin as indicated by clonogenic survival assays. SALL4 KD and control cells were treated with 0.1 μM Doxorubicin (Dox) for the indicated time points, cultured for 2 weeks, and stained with crystal violet solution. Survival fraction is calculated by comparing the plating efficiency (PE: colony numbers/seeding numbers). $N = 3$ biological repeats. Two-way ANOVA followed Bonferroni's multiple comparisons test. (**d**) DDR was impaired in SALL4 KD cancer cells. SALL4 KD cells and control cells were exposed to 0.5 μM Dox for indicated time points and analyzed for the levels of ATM-S1981p, ATM, KAP1-S824P, KAP1, γ-H2AX, H2AX, acetylated histone H3 lysine 14 (H3K14ace), tri-methylated H3K9 (H3K9me3), histone H3 (H3), SALL4 and tubulin. The ratio of the levels of phosphorylated form to the total protein levels was indicated. (**e**) SALL4 KD cells and control cells were treated with 0.5 μM Dox for the indicated times, and analyzed for the levels of ATM-S1981P, KAP1-S824P, γ-H2AX, H3K14ace and SALL4 by IF. Fixed cells were immunostained with indicated antibodies. DNA was counterstained with DAPI (blue). Scale bar, 10 μm. For quantification, the fluorescence intensity was measured by ImageJ and displayed as arbitrary unit (A.U.) in the graphs. For statistic analysis, two-tailed unpaired *t*-tests were used as statistical analysis. $N = 29$ individual cells for each cell line. For the analysis of phosphorylated ATM and γ-H2AX, One-way ANOVA followed by Sidak's multiple comparisons. $N = 30$ individual cells for each cell line. Rr is the Pearson's correlation coefficiency for analyzing the co-localization of SALL4 with γ-H2AX, and a value of one indicates complete co-localization. In response to DNA damage, the co-localization of SALL4 with γ-H2AX is increased. $N = 14$ individual cells for each cell line. Scale bar is 10 μm. (**f**) SALL4 KD cells are defective in DNA repair as indicated by the Comet assay. Cells were treated with 0.5 μM Dox for 4 h and subsequently incubated with normal media for 16 h. One-way ANOVA followed by Sidak's multiple comparisons test. $N = 43$ individual cells. Scale bar is 50 μm. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Error bars indicate means ± standard deviation (s.d.).

intensity of SALL4 and HP1α in the nucleus, suggesting that SALL4 might destabilize HP1α (Figure 2a, last panels). In support of this notion, we detected an interaction between SALL4 and HP1α that could be stabilized by the treatment with proteinase inhibitor (Figure 2b). In further support of a role of SALL4 in regulating the protein stability of HP1α, the depletion of SALL4 in cancer cells increased the protein levels of HP1α, but not those of HP1β and HP1γ (Figure 2c) and the overexpression of SALL4 reduced the protein levels of HP1α (Figure 2d). Considering that SALL4 is a transcription factor, the levels of HP1α mRNA in SALL4 KD cells and control cells were examined, indicating that SALL4 does not regulate the mRNA levels of HP1α (Figure 2e). To directly test

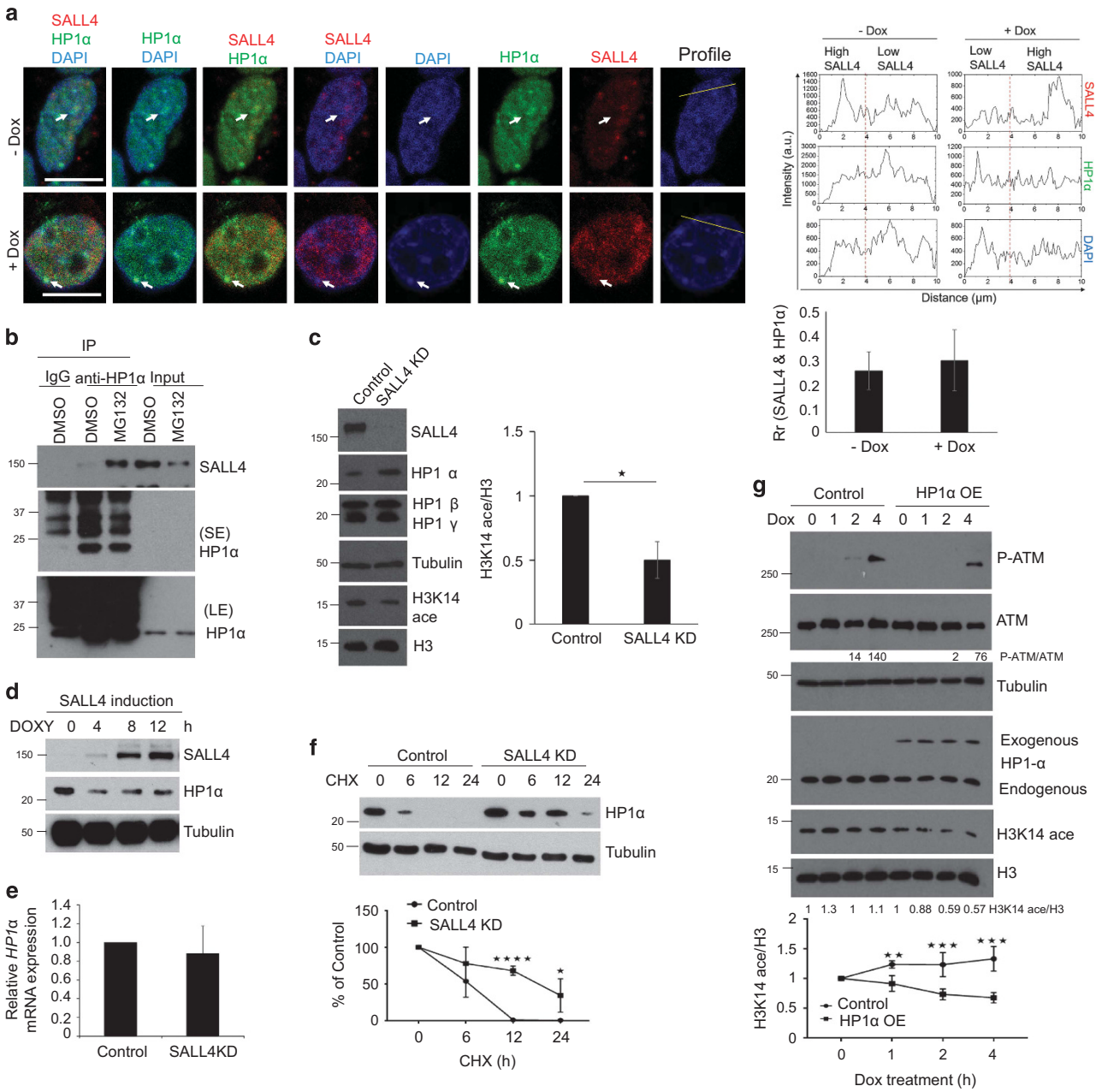


Figure 2. SALL4 reduced the stability of HP1α. (**a**) SALL4 was modestly co-localized with HP1α. SNU-398 cells were treated with 0.5 μM Dox for 4 h, fixed, and immunostained with the indicated antibodies. DNA was counterstained with DAPI (blue). Rr is the Pearson's correlation coefficient, and a value of one indicates complete co-localization. Scale bar is 10 μm. The intensity of the fluorescence was analyzed by ImageJ. (**b**) The association between SALL4 with HP1α was increased by the treatment with proteinase inhibitor. Nuclear fractions of SNU-398 cells either mock treated or treated with 5 μM proteasome inhibitor MG132 for 12 h were subjected to immunoprecipitation (IP) with anti- HP1α antibody. The protein levels of SALL4 and HP1α in the immunoprecipitate and in the input were determined. SE and LE, short and long exposure of the same gel. (**c**) The silencing of SALL4 in cancer cells led to increased protein levels of HP1α and reduced levels of H3K14 acetylation (H3K14ace). Images are representative of three biological repeats. The acetylation levels of H3K14 were normalized by the total histone 3 (H3) levels. (**d**) The protein levels of SALL4 in HepG2 cells were inversely correlated with the protein levels of HP1α. Cells were transduced with SALL4 inducible expression lentiviral vector and the expression of SALL4 was induced by Doxycyclin (DOXY). (**e**) Silencing of SALL4 did not affect the HP1α mRNA levels as indicated by qPCR. $N = 4$ biological repeats. (**f**) Silencing of SALL4 significantly increased the stability of HP1α protein. SALL4 KD cells and control cells were treated with cycloheximide (CHX) for indicated time, and analyzed for the protein levels of HP1α and tubulin. For quantification, the band intensity of HP1α was normalized by tubulin. $N = 3$ biological repeats. (**g**) The overexpression of HP1α suppressed ATM activation and the H3K14 acetylation. HepG2 cells were transfected with empty vector or vector expressing HP1α, and treated with Dox for indicated times and analyzed by western blotting to detect ATM-S1981p, ATM, HP1α, H3K14ace and tubulin. For quantification, the ratio of the band intensity of phosphorylated form of ATM and total ATM is shown. $N = 4$ biological repeats. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Error bars indicate means ± s.d. Two-way ANOVA followed by Sidak's multiple comparisons (f, g).

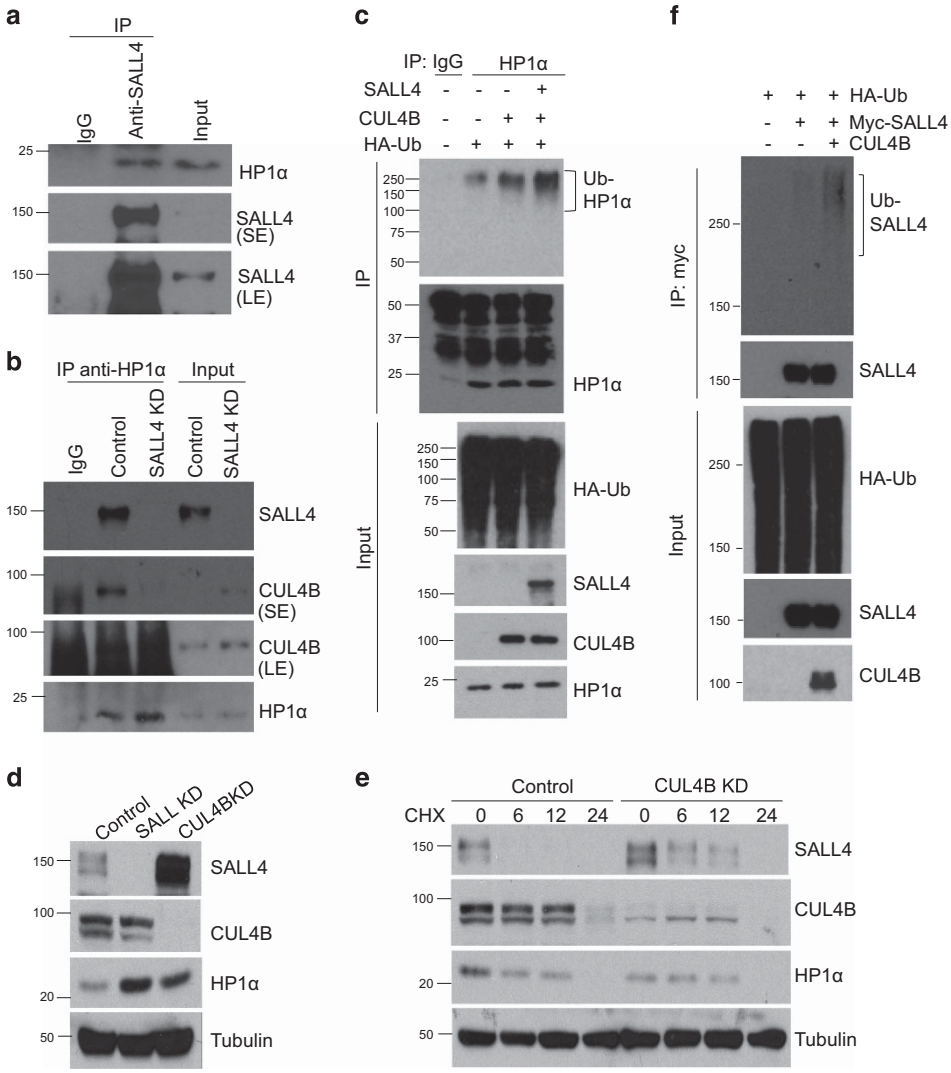


Figure 3. SALL4 recruits CUL4B to HP1α to ubiquitinate HP1α. (**a**) SALL4 interacted with HP1α as determined by co-IP. Nuclear fraction of SNU-398 cells was subjected to IP with anti-SALL4 antibody. The presence of SALL4 and HP1α in the immunoprecipitate and in the input was determined. SE and LE, short and long exposure of the same gel. (**b**) The interaction between CUL4B and HP1α was dependent on SALL4. Nuclear fraction of SALL4 KD cells and control cells were subjected to IP with anti-HP1α antibody. The input and the immunoprecipitate were subjected to western blotting with anti-SALL4, anti-CUL4B, and anti-HP1α antibodies. SE, short exposure; LE, long exposure. (**c**) SALL4 promoted the CUL4B-dependent ubiquitination of HP1α. 293T cells transfected with indicated expression vectors were lysed and subjected to IP with anti-HP1α antibody. The resulting immunoprecipitates and the input were subjected to western blotting with antibodies to SALL4, CUL4B and HA. (**d**) The silencing of CUL4B increased the protein levels of HP1α and SALL4. SALL4 KD cells, CUL4B KD cells, and control cells were analyzed for protein levels of SALL4, CUB4B, HP1α and tubulin. (**e**) The silencing of CUL4B increased the stability of SALL4 and HP1α. CUL4B KD and control cells were treated with CHX for the indicated time and analyzed for the expression of SALL4, HP1α, CUL4B and tubulin. (**f**) CUL4B was an E3 ligase for SALL4. 293T cells transfected with indicated expression vectors were lysed and subjected to IP with anti-myc antibody. The resulting immunoprecipitate and the input were subjected to western blotting with antibodies to SALL4, CUL4B and HA.

whether SALL4 regulates the protein stability of HP1α, we examined the stability of HP1α in SALL4 KD and control cells by determining the protein levels of HP1α after the treatment with cycloheximide (CHX), indicating that SALL4 decreases the protein stability of HP1α (Figure 2f).

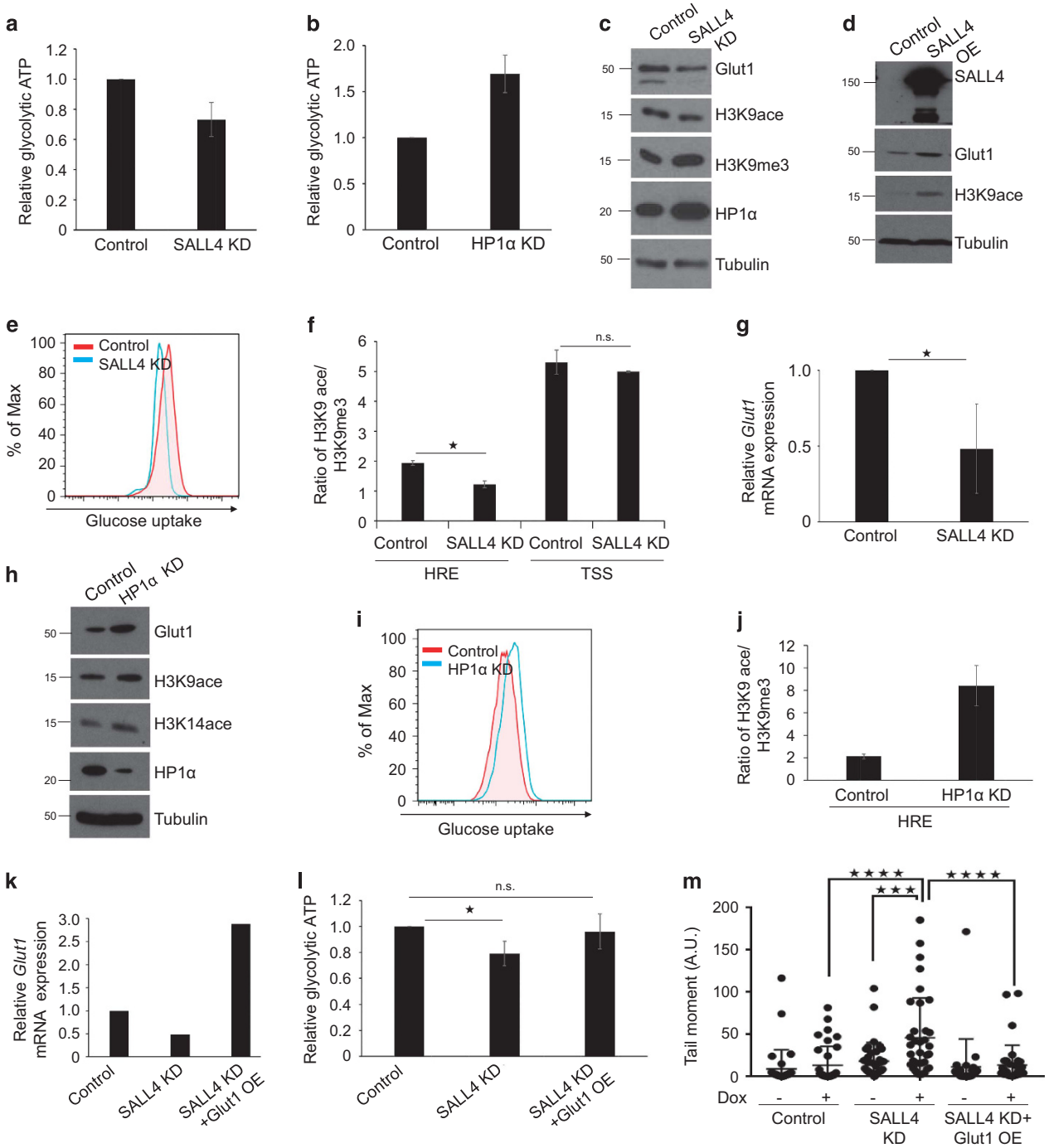
Open chromatin structure permits the access of DNA repair machinery to the site of DNA damage and thus enhances the DDR signaling pathway, and compact chromatin structure limits the extension of γ-H2AX from site of DNA damage in activating DDR.[28-31] Considering the importance of HP1α in regulating chromatin epigenetics, we analyzed the change in the chromatin epigenetics in SALL4 KD and control cells in response to DNA DSB damage. When compared to the control cells, the levels of acetylated H3K14 (H3K14ace), which are increased at the DNA damage sites to activate DDR,[28,32] were decreased in SALL4 KD cells in response to DNA damage (Figure 2c). Recent findings that chromatin decondensation caused by HP1α deficiency abrogates the accumulation of DNA damage in ATM-deficient cells suggest that the chromatin relaxation due to the reduced HP1α protein levels is important for DDR.[33] The overexpression of HP1α impaired the activation of ATM and the induction of histone acetylation after DNA damage (Figure 2g). Therefore, the increased levels of HP1α could contribute to the impaired DDR and chromatin remodeling in SALL4 KD cells.

SALL4 is important for embryonic stem cells that share the open chromatin structure with cancer cells. Therefore, we examined whether SALL4 plays the same roles in regulating chromatin epigenetics in ESCs. By silencing the expression of SALL4 in human embryonic stem cells (hESCs), we demonstrated that the depletion of SALL4 in hESCs also increased the protein levels of

HP1α and reduced the chromatin openness (Supplementary Figure 3). Therefore, SALL4 plays the same role in maintaining chromatin openness in hESCs as in cancer cells.

SALL4 promotes HP1α ubiquitination by recruiting E3 ligase CUL4B

Using co-immunoprecipitation, we demonstrated that SALL4 interacted with HP1α (Figures 2b and 3a). We hypothesized that SALL4 could recruit E3 ligase to HP1α to destabilize HP1α, and examined the list of proteins associated with SALL4 we previously identified by mass spectrometry (MS).[26] We focused on the candidates involved in regulating protein stability and identified one candidate Cullin4B (CUL4B). CUL4B is one of the core components of Cullin4B-Ring E3 ligase complex (CRL4B).[34] In contrast to other Cullins, CUL4B is localized in the nucleus via nuclear localization signal (NLS), suggesting that CUL4B has roles in the nucleus. In support of the notion that SALL4 can recruit CUL4B to HP1α, co-immunoprecipitation studies showed the interaction among SALL4, CUL4B and HP1α, and the interaction was dependent on SALL4, as the depletion of SALL4 from the cells disrupted the association between CUL4B and HP1α (Figure 3b). In addition, SALL4 promoted the ubiquitination of HP1α by CUL4B (Figure 3c), and the depletion of CUL4B significantly increased the protein levels and stability of HP1α (Figures 3d and e). Together, these data demonstrate that SALL4 recruits CUL4B to HP1α, leading to the ubiquitination and degradation of HP1α. The



depletion of CUL4B also led to increased protein levels of SALL4 and the stability of SALL4, indicating that CUL4B is also an E3 ligase for SALL4 (Figures 3d and e). In support of this notion, CUL4B could promote the ubiquitination of SALL4 (Figure3f).

SALL4-HP1α pathway promotes glycolysis and DDR

It has been well established that open chromatin promotes DDR.[35] Recent studies also support the notion that glycolysis promotes the open chromatin structure, because glycolytic metabolites such as acetyl-coA and lactate provide the substrates to maintain the elevated levels of histone acetylation in cancer cells, leading to the resistance to chemotherapy drugs.[36,37] Therefore, we test the hypothesis that SALL4-HP1α axis is involved in promoting glycolysis in cancer cells and thus maintain open chromatin structure. In support of this notion, the knockdown of SALL4 decreased the glycolytic ATP generation, while the depletion of HP1α increased it (Figures 4a and b). To understand the mechanism how SALL4 promotes glycolysis, we discovered that the knockdown of SALL4 decreased the expression of Glut1 (Figure 4c), a key protein involved in glycolysis.[38] In addition, the overexpression of SALL4 increased the levels of Glut1 and acetylated H3K9, consistent with the correlation between glycolysis and open chromatin (Figure 4d). Consistent with these findings, the glucose uptake was decreased in SALL4 KD cells compared to control cells (Figure 4e). In addition, overexpression of SALL4 increased lactate production (Supplementary Figure 4). These data indicate that SALL4 induces the expression of Glut1 in cancer cells, and thus promoting glycolysis and drug resistance.

It has been established that the expression of Glut1 is regulated by hypoxia inducible factor 1α (HIF-1α) through the hypoxia responsive elements (HREs) within the promoter and is controlled by the acetylation of H3K9 in the promoter.[39,40] To determine how the depletion of SALL4 affects H3K9me3 and H3K9ace in the HRE of Glut1 promoter, chromatin immunoprecipitation (ChIP) was performed to indicate lower H3K9ace and higher H3K9me3 within HRE in the promoter of Glut1 in SALL4 KD cells when compared to control cells, but not at transcriptional start site (TSS), leading to the reduction of Glut1 mRNA levels (Figures 4f and g).

To test whether HP1α is involved in suppressing Glut1 expression in SALL4 KD cells, we examined the impact of the silencing of HP1α on GLUT1 expression. Our data indicated that the depletion of HP1α increases both GLUT1 expression and H3K14ace (Figure 4h) as well as glucose uptake (Figure 4i), supporting the notion that HP1α suppresses the expression of Glut1 by reducing H3K14ace. In addition, the depletion of HP1α led to increased H3K9ace and reduced H3K9me3 within HRE in the promoter of Glut1, providing the mechanism how SALL4 promotes the expression of GLUT1 by destabilizing HP1α (Figure 4j).

To determine the importance of SALL4-HP1α-GLUT1 pathway in DDR of cancer cells, we examined DDR in SALL4 KD cells with restored GLUT1 expression (Figure 4k). The restoration of Glut1 expression rescued the decrease of glycolytic ATP generation in SALL4 KD cells (Figure 4l). While SALL4 KD cells were less efficient in repairing DNA DSB damage than control cells, the restoration of GLUT1 protein levels effectively rescued such defects, supporting the importance of SALL4-HP1α-GLUT1 pathway in promoting drug resistance in SALL4-expressing cancer cells (Figure 4m).

DISCUSSION

Accumulating data have indicated that SALL4 promotes drug resistance of cancer cells. We discovered a novel mechanism underlying the SALL4-mediated DDR and DNA repair. In contrast to the popular assumption that the heterochromatin-associated SALL4 is a transcription repressor,[41] we demonstrated that SALL4 recruits E3 ubiquitin ligase CUL4B to HP1α, leading to the degradation of HP1α and open chromatin. In this context, the importance of HP1α in DDR and DNA repair has been supported by the findings that HP1α deficiency can rescue the impairment of DDR in $ATM^{-/-}$ cells by relaxing chromatin to promote DNA repair.[29] The roles of SALL4 in regulating open chromatin and DDR are conserved between embryonic stem cells and cancer cells, further supporting a functional link between cancer and pluripotency.

Together with the findings that SALL4 promotes the ATM activation by stabilizing MRE11 complex at the site of the DNA damage,[26] these studies demonstrate multiple roles of SALL4 in promoting DNA repair. In this context, the SALL4-mediated destabilization of HP1α promotes glycolysis that contributes to chemo-drug resistance. Numerous studies have proposed that the expression levels of HP1α play important roles in cancer development. Low levels of HP1α have been associated with the invasive metastatic cancers including breast and colon cancers, and the downregulation of HP1α is critical for metastasis in those cancers.[42] Therefore, we speculate that the SALL4-HP1α interaction could be a critical pathway not only for promoting drug resistance but also for cancer metastasis.

Figure 4. SALL4 induces the expression of Glut1 and glycolysis by destabilizing HP1α. The knockdown of SALL4 inhibited glycolysis (**a**), and the knockdown of HP1α promoted glycolysis (**b**). Glycolytic ATP production was assessed by the treatment with glycolysis inhibitor 2-deoxyglucose (2-DG). To exclude the amount of ATP produced by mitochondrial oxidative phosphorylation, the cells were also treated with oligomycin, a potent inhibitor of mitochondrial ATP synthase. $N = 2$ biological repeats. (**c**) The knockdown of SALL4 inhibited the expression of Glut1 and chromatin openness. The SALL4 KD cells and control cells were analyzed by western blotting for the expression of Glut1, H3K9ace, H3K9me3, HP1α, Glut1 and tubulin. (**d**) The overexpression of SALL4 increased the expression of Glut1 and chromatin openness. HepG2 cells were transfected with empty vector or vector expressing SALL4, and 36 h after transfection, cells were analyzed for the expression of Glut1, SALL4, H3K9ace and tubulin. (**e**) SALL4 KD and control cells were incubated in HBSS with the fluorescent glucose analog 2-NBDG for 15 min, and glucose uptake was then quantified using flow cytometry (FACS). Median fluorescence intensity (MFI) of control cells and SALL4 KD cells are 2308 and 1476, respectively. Data shown are representative of two biological repeats. (**f**) The knockdown of SALL4 reduced the ratio of H3K9ace versus H3K9me3 within HRE of the promoter of the Glut1 gene as determined by chromatin-IP (ChIP)–qPCR. Promoter region around hypoxia responsive element (HRE) site and region around transcriptional start site (TSS) were analyzed. $N = 3$ biological repeats. (**g**) Real-time RT-PCR analysis of Glut1 mRNA expression in SALL4 KD cells and control cells. Error bars indicate means ± s.d. $N = 5$ biological repeats. (**h**) The knockdown of HP1α increased the protein levels of Glut1, as well as H3K9ace and H3K14ace. (**i**) The knockdown of HP1α increased the glucose uptake. MFI of control cells and HP1α KD cells are 1350 and 2134, respectively (representative for two biological repeats). (**j**) The knockdown of HP1α increased the ratio of H3K9ace versus H3K9me3 within HRE of the promoter of the Glut1 gene. $N = 2$ biological repeats. (**k**) Restoration of the Glut1 expression in SALL4 KD cells. $N = 2$ biological replicates. (**i**) Glycolytic ATP production was restored by the re-expression of Glut1 in SALL4 KD cells. $N = 4$ biological repeats. (**m**) The restoration of Glut1 expression in SALL4 KD cells rescued the defects in DNA repair. Cells were treated with Dox for 4 h and further incubated with normal media for 16 h. $N = 26$ (SALL4 KD+Glut1 OE without Dox treatment) or 36 (all others) individual cells. * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$. One-way ANOVA followed by Bonferroni multiple comparison test (**f**, **l**) or Sidak's multiple comparisons (**m**). Two-tailed paired *t*-test (**g**). Error bars indicate means ± s.d.

MATERIALS AND METHODS

Cell lines, viral vectors, and transfection

All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). To establish cell line with knockdown of SALL4 and hHP1α, DNA fragments for short hairpin RNA (shRNA) and control scramble shRNA were cloned into the pLKO.1 lentiviral vector. The sequences are listed as following: shSALL4 5′-CCGGAGCTATTTAGCCAA AGGCAAA CTCGAG TTTGCCTTTGGCTAAATAGCTTTTTG-3′; shSALL4 5′-AATTCAAAAAAGCTATTTAGCCAAAGGCAAACTCGAGTTTGCCTTTGGCTAA ATAGCT-3′; Scr shRNA 5′-CCGGGGGTACGGTCAGGCAGCTTCTCTCGAGA GAAGCTGCCTGACCGTACCCTTTTTG-3′; Scr shRNA 5′-AATTCAAAAAGGGTAC GGTCAGGCAGCTTCTCTCGAGAGAAGCTGCCTGACCGTACCC-3′; hHP1α 5′-C CGGAACAAGAGGAAATCCAATTTCCTCGAGGAAATTGGATTTCCTCTTGTTTTT TTG-3′; hHP1α 5′-AATTCAAAAAAACAAGAGGAAATCCAATTTCCTCGAGGA AATTGGATTTCCTCTTGTT-3′

Using the calcium phosphate method, lenti-viruses were produced by co-transfecting HEK293T cells with the lentiviral vectors and packaging vectors as previously described.[26] Three days after infection, lenti-virus was collected and concentrated by Lenti-X concentrator (Clonetech, Mountain View, CA, USA), and was used to transduce cells in 10 μg/ml polybrene. For establishing HepG2 cells with inducible expression of SALL4, FUW-TetO SALL4 lenti-virus and FUW-M2rtTA (Addgene plasmid 20342) lenti-virus were produced to infect cells, which were selected with 200 μg/ml Zeocin for 14 days. To overexpress Glut1 in SALL4 KD cells, Glut1 cDNA-IRES was inserted in front of puromycin resistant gene of PLKO1-shSALL4 plasmid. To express human SALL4 or HP1α, the full-length cDNA of SALL4 or HP1α were inserted into pCDNA3 or eGFPN1 vector, respectively.

DNA damage assay

The sensitivity of human cancer cells to doxorubicin was examined by the clonogenic assay as previously described.[43] One day after plating the cells (2000 cells/well), the cells were treated with 0.1 μM Dox for 1 h or 2 h and cultured for 2 weeks, stained with crystal violet and counted. After the cells were treated with 0.5 μM Dox for 4 h and further incubated without Dox for 16 h, Comet assay was used to examine the levels of DNA DSB damage with a kit (Cell Biolabs, San Diego, CA, USA).

Immunofluorescence microscopy analysis

Cells grown on a chamber slide were treated with 0.5 μM Dox for 4 h, fixed with 4% paraformaldehyde solution (Affymetrix, Santa Clara, CA, USA) for 15 min at room temperature, permeabilized with 0.3% Triton X-100, and stained with indicated antibodies overnight at 4 °C. The images were acquired with confocal microscope (FV1000; Olympus) and quantified with ImageJ software as previously described.[26]

Protein analysis

Total proteins were extracted with lysis buffer or by directly adding SDS sample buffer as previously described.[26] To perform co-immunoprecipitation (Co-IP) of SALL4, HP1α and CUL4B, 1 mg nuclear extract prepared from SALL4 KD cells and control cells was immunoprecipitated as previously described,[44] with polyclonal antibody against HP1-α (GTX63394) or monoclonal antibody against SALL4 (Abcam; 57577). For protein ubiquitination analysis, 293T cells transfected with various combinations of vectors expressing HA-Ub, SALL4 and CUL4B were lysed with lysis buffer supplemented with phosphatase inhibitor and protease inhibitor cocktails, and immunoprecipitated with monoclonal anti-HA antibody (Covance; MMS-101 R). The antibodies used were described previously.[26] The intensity of protein bands was quantified using ImageJ software (National Institutes of Health).

ChIP assay was performed as previously described.[26] The levels of DNA purified from the immunoprecipitated chromatin were determined by quantitative real-time PCR. The sequence of the primers is the following: HRE 5′ CTCCTGGAGTCTCTCTAACA 3′, HRE 5′ GGGATAGTAACAG TACCACC 3′, TSS 5′ AAACCTTTCCTCCTGGCTGC 3′, TSS 5′ AGCTCCAGA GTTAGGAGTGAGT 3′.

Glucose uptake and ATP production

Cells were incubated with HBSS with 30 μM 2-NBDG (Cayman) for 15 min, washed with HBSS, and analyzed by flow cytometry as described previously.[2] To determine the levels of glycolytic and oxidative ATP production, cells were incubated in oligomycin (1 μM, Sigma) to suppress oxidative ATP generation or 2-deoxy-D-glucose (20 mM/m Sigma) to suppress glycolytic ATP production for 2 h. Luminescence was measured with NOVOstar (BGM Labtech., Cary, NC, USA).

Quantitative PCR analysis

Total RNA extraction and quantitative PCR analysis were performed as previously described.[2] The primers used were following: actin 5′-CAGA GCCTCGCCTTTGCCGATC-3′; actin 5′-CATCCATGGTGAGCTGGCGGCG-3′; Glut1 5′-CTTGGCTCCCTGCAGTTTG-3′; Glut1 5′-GGACCCATGTCTGGTTGT AG; HP1α 5′-CGATCCGGTAGGTAAAGGAGG-3′; HP1α 5′-TGCCTCACCAGGA AAAACAAGG-3′.

Statistical analysis

Any statistical method was used to calculate sample size and Group variation was not estimated before experiments. Statistical significance was assayed with GraphPad Prism and excel. For comparing two groups, *t*-test was used. F-test was tested for comparing variances. For the multiple comparisons, data were first subjected to one-way ANOVA or two-way ANOVA, followed by Sidak's or Bonferroni's multiple comparisons test to determine statistical significance. $^{\star}P < 0.05$; $P < 0.01$; $P < 0.001$; $P < 0.0001$; n.s., not significant. Error bars indicate means ± s.d.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

J.K. with the help of S.X. and L.Y. performed experiments. J.K, X.F. and Y.X. planned the experiments and interpreted the data. X.F. and Y.X. provided the administrative support. J.K. and Y.X. were responsible for the initial draft of the manuscript, whereas other authors contributed to the final edited versions.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)