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FOXA1 antagonizes EZH2-mediated CDKN2A repression in carcinogenesis

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ABSTRACT

CDKN2A ($p16^{INK4a}$) is a crucial tumor suppressor involved in many cancers. Our recent investigations revealed that FOXA1 as a forkhead transcription factor mediates CDKN2A activation in cellular senescence. However, the contribution of this axis in carcinogenesis remains unclear. Here, using a comprehensive collection of cancer microarray data, we found FOXA1 is down-regulated in many cancers compared to their normal counterparts and the positive correlation between FOXA1 and CDKN2A could be observed in prostate and breast cancers with lower EZH2 (epigenetic repressor for CDKN2A) expression. Experimentally, epistasis analysis in prostate and breast cancer cells indicated that higher expression of FOXA1 opposes EZH2-mediated CDKN2A repression, as further depletion of FOXA1 reverts the de-silencing of CDKN2A caused by EZH2 inhibition. Concomitantly, EZH2-depletion suppresses cancer cell cycle progression and this regulation is optimized in the presence of FOXA1 and CDKN2A. A further oncogenic transformation assay suggested that overexpression of EZH2 is insufficient to block RAS-induced CDKN2A activation and loss of FOXA1 is mandatory to potentiate EZH2-mediated CDKN2A silencing and to bypass the senescence barrier. Importantly, using an *in vitro* histone methyltransferase (HMTase) system, we found FOXA1 directly inhibits EZH2's histone methyltransferase activity through its C-terminal histone binding motif. These data support that positive regulation of CDKN2A by FOXA1 counteracts its tumorigenic repression of by EZH2 in cancers.

1. Introduction

Epigenetic deregulation including DNA hypermethylation and aberrant repressive histone modifications is frequently observed at *cis*-regulatory elements of tumor suppressor genes in carcinogenesis [1]. As key regulators of G1 phase cell-cycle arrest and senescence, *CDKN2A*/*CDKN2B*/*ARF* on chromosome 9p21 is inactivated in a wide range of human cancers through epigenetic mechanisms [2]. Two major Polycomb group (PcG) protein complexes, namely Polycomb Repressive Complex 1(PRC1) and Polycomb Repressive Complex 2 (PRC2) are implicated in transcriptional repression of CDKN2A [3]. The PRC2 complex deposits methyl groups on histone H3 at lysine 27 (H3K27me3) by its methyltransferase component EZH2 and consequently inhibits downstream gene transcription by collaborations with the PRC1 complex [4]. In line with their repressor functions on CDKN2A, EZH2 is up-regulated in several cancers associated with poor prognosis and is indicative of a metastatic potential of later tumor development [5].

Our functional investigations on transcription factors (TFs) with a potential to activate CDKN2A in cellular senescence revealed that FOXA1 is a key TF for CDKN2A expression [6]. FOXA1 is a member of forkhead family TFs with remarkable pioneering activity to open closed chromatin for its subsequent cooperation with other master TF in embryogenesis and organ development [7,8]. This distinctive feature of FOXA1 to open chromatin was mediated by its DNA binding pocket and by the C-terminal domain which directly binds histones H3 and H4 [9]. In senescence, FOXA1 binds to its *cis* regulatory element at the promoter region of CDKN2A, stimulates nucleosome remodeling and engages a long-range promoter–enhancer communication to initialize CDKN2A expression [6]. Importantly, antagonisms between FOXA1 and PRC2 on the histone tail modifications and chromatin structure at CDKN2A promoter determine the final outcome of CDKN2A in both replicative exhaustion and oncogene induced senescence [6].

On the other hand, oncogenic property of FOXA1 is demonstrated in hormone-dependent cancers, such as oestrogen receptor (ER)-positive breast cancer and androgen receptor (AR)-positive prostate cancer [7]. In ER mediated cancers, FOXA1 binds to

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approximately 50% of all ER-binding regions and contributes to the preparation of optimal chromatin conditions for ER recruitment [10,11]. However, down-regulation of FOXA1 was also proposed to be an unfavorable prognostic signature in progressive prostate cancer with genomic redistribution of AR and with aberrant cell cycles, implying a negative role of FOXA1 for cancer exacerbation [12,13].

Given by the intimate link between senescence and tumor regression [2], it merits an in-depth analysis for FOXA1's status in cancer development in view of our findings on the transcriptional regulation of CDKN2A by FOXA1. In this manuscript, we performed *in silico* computational survey and *in vivo* epistasis analysis to evaluate the role of FOXA1 and EZH2 to regulate CDKN2A expression and to test their relevance for carcinogenesis. These results revealed a novel senescence-barrier function of FOXA1 in cancers which inhibits PRC2-catalyzed histone H3 K27 trimethylation at CDKN2A promoter enzymatically through its C-terminal histone binding motif.

2. Materials and methods

2.1. Databases and gene expression data

FOXA1 gene expression in normal or malignant human tissues was obtained from the Oncomine database (http://www.oncomine.org) [14]. Differential expression levels of FOXA1 across various tumor types in Fig. 1 were produced from Oncomine's online differential analysis tool. Scatterplot and boxplot analysis were conducted in R (http://www.r-project.org) with data download from Oncomine. Comparison of FOXA1 expression between samples was performed only when gene expression data was from a same study, using the same methodology. For the correlation analysis in Fig. 1, the normalized gene expression was retrieved from Gene Expression Omnibus [15–17].

2.2. Anchorage-independent transformation assay

Cells (2×10^4 cells/well) were seeded in 6-well plates in DMEM medium containing 0.35% low melting point (LMP) agar overlying a 0.7% LMP agar layer. Plates were incubated in 5% CO_2 at 37 °C for 2–3 weeks. Colonies were then counted and images were captured on an Olympus MT2 microscope.

2.3. Histone binding assay

For a FOXA1-dependent histone binding experiment, purified full-length (FL), C-terminal-deleted (ΔCt), and DBD-deleted (ΔBDD) FOXA1 proteins were first immobilized on FLAG-M2 resin. The resin was then incubated with 2 μg reconstituted mononucleosome for 4 h in 4 °C. After pelleting the beads, the supernatant was boiled in SDS sampling buffer; the beads were washed extensively and the bound fraction was also collected in SDS sampling buffer. These supernatant and bound fractions were subjected to Western blotting analysis with anti-H3 antibody.

2.4. Histone methyltransferase (HMT) assay

In a standard HMT assay, 0.5 μg or 1 μg of recombinant human PRC2 complex were used to provide methyltransferase activity and 2 μg of oligonucleosomes purified from HeLa cells were used as substrates. The reaction was performed in a 50 μl reaction volume with HMT buffer (20 mM Tris–HCl, 4 mM EDTA, 1 mM PMSF, 0.5 mM DTT, pH 7.9) and 0.3 μm S-adenosyl-L-methionine for 1 h at 30 °C. When indicated in Fig. 4, 0.5 μg of full-length or deletion-mutated FOXA1 proteins were included in HMT assay. C-terminal peptide of FOXA1 (IEPSALEPAYYQGVYSRPVLNTS) was synthesized in MBL. When indicated, 0.25 μg FLAG peptide or FOXA1-Ct peptide was included in HMT assay.

Detailed descriptions for cell culture, plasmid and RNAi sequences, protein purification, and cell cycle analysis were provided in the online SupplementaL text.

3. Results

3.1. Regulation of CDKN2A by FOXA1 and EZH2 in cancers

A meta-analysis was firstly performed to evaluated the expression of FOXA1 in various cancers with public Oncomine database [14]. We retrieved all the cancer samples from Oncomine in which the differential expression of FOXA1 in cancer versus its normal control counterpart passes three thresholds: fold change (FC) > 2 or <0.5, *p*-value <1E-4 (Student's *t*-test), and FOXA1'rank is within top 10% changed genes. This analysis revealed significant enrichment of FOXA1 in breast and prostate cancers, in addition to bladder and lung cancers consistent with previous reports [7] (Figs. 1A and S1A). However, there is more number of tumor types with significant FOXA1 down-regulation including brain, colorectal, gastric, liver, skin, lymphoma and pancreatic cancers (Fig. S1A). The scatterplot clearly demonstrated this point as the samples meeting the filter criteria were dispersed into two equal-sized groups with either substantial gain or loss of FOXA1 expression (Fig. 1A). Significant FOXA1 reduction was also exemplified in specific samples, such as in rectal adenocarcinoma ($n = 237$, $p = 2.64E\text{-}14$) (The Cancer Genome Atlas), tongue squamous cell carcinoma ($n = 58$, $p = 5.26E\text{-}6$) [18], hepatocellular carcinoma ($n = 115$, $p = 9.49E\text{-}11$) [19], cutaneous follicular lymphoma ($n = 16$, $p = 3.91E\text{-}6$) and marginal zone B-cell lymphoma ($n = 13$, $p = 1.55E\text{-}5$) [20], and melanoma ($n = 52$, $p = 5.92E\text{-}6$) [21] (Fig. 1B).

Given by the critical role of FOXA1 in steroid hormone related cancers [7], we then performed a detailed analysis on the correlation of FOXA1 and CDKN2A in prostate and breast cancers. Using microarray data from one prostate cancer study [16] ($n = 30$), we observed positive correlation between FOXA1 and CDKN2A across the whole dataset (Fig. 1C Pearson's correlation coefficient = 0.37, $p = 0.04601$). However, investigation on another prostate cancer dataset [17] failed to reveal a significant correlation between these two genes, possibly due to the genetic and epigenetic heterogeneity of cancers. Consequently, in view of the established transcriptional repression of CDKN2A by EZH2 [3], we refined the correlation analysis in the same dataset on basis of differential EZH2 expression. Indeed, positive correlation between FOXA1 and CDKN2A was specifically observed in the stratified subgroups with lower EZH2 expression but not in groups with higher EZH2 level (Figs. 1D and S1B). We then tested this pattern in a large dataset with microarray data from 204 breast cancer samples [15]. Consistently, significant positive correlation could only be observed in subgroups with lowest EZH2 expression (Figs. 1E and S1C). The positive correlation specifically observed in tumor samples with low expression of EZH2 implies a counteracting mechanism for the control of CDKN2A expression in cancer development by FOXA1-dependent activation and PRC2-dependent repression.

3.2. Loss of FOXA1 potentiates EZH2-mediated epigenetic repression of CDKN2A

To experimentally validate the results from *in silico* analysis, we performed an epistasis analysis in LNCaP prostate cancer cells, which bear high level of EZH2 and FOXA1 [13]. In concert with

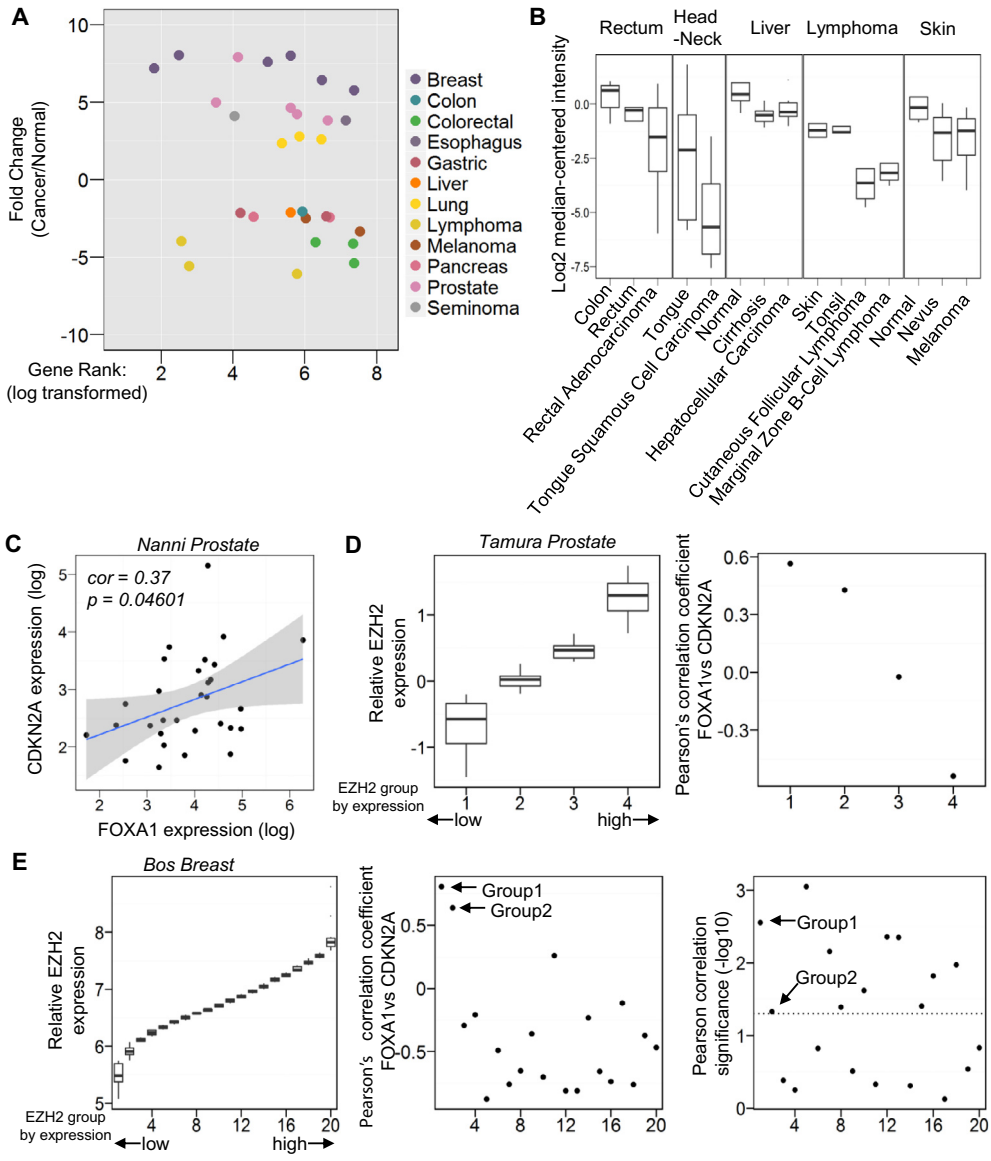
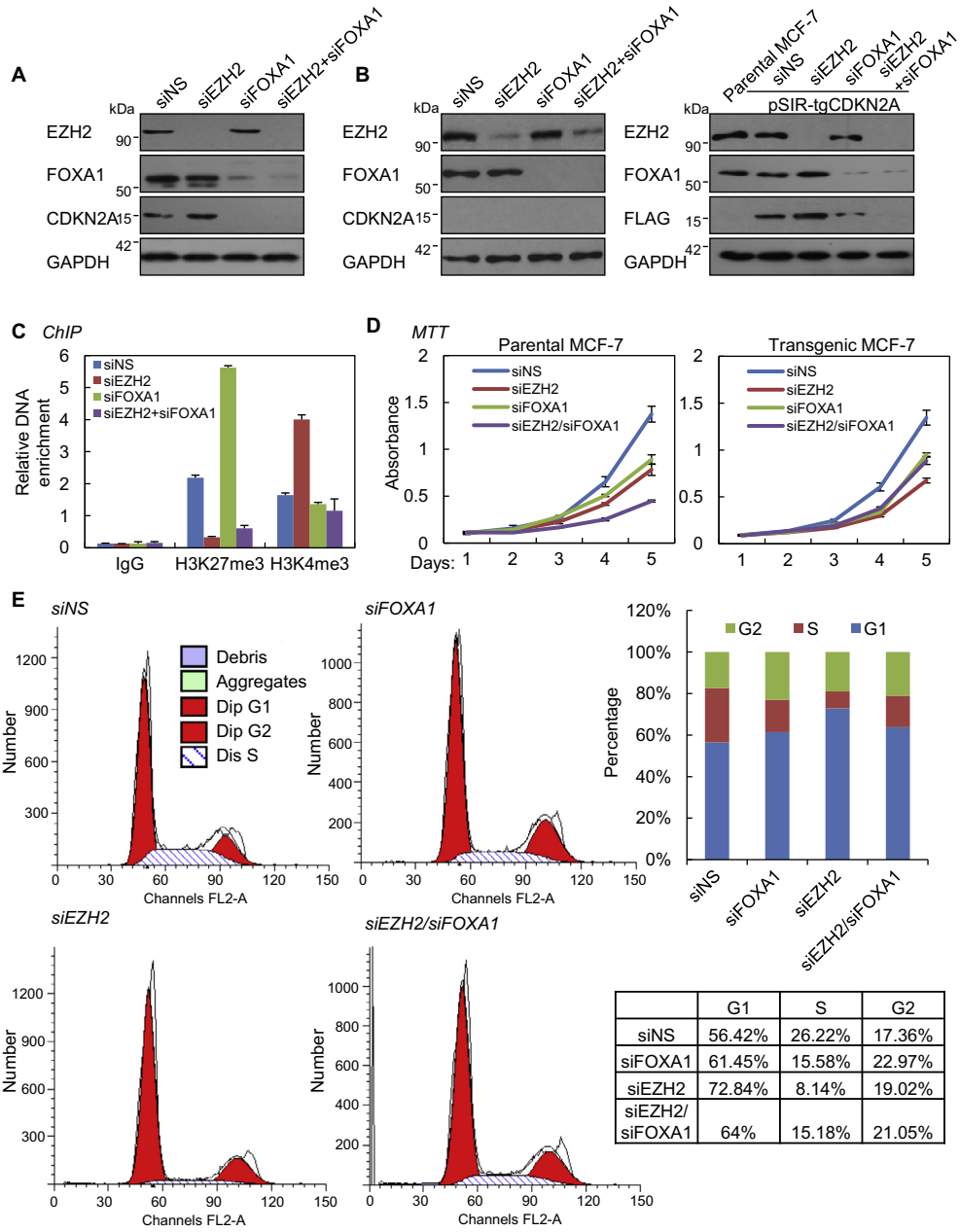


Fig. 1. Regulation of CDKN2A by FOXA1 and EZH2 in cancers. (A) Variation of FOXA1 expression in different cancer specimens. We collected all the cancer samples from Oncomine [14] in which the differential expression of FOXA1 in cancer versus its normal control counterpart passes three thresholds: fold change (FC) > 2 or <0.5, *p*-value <1E-4 (*t*-test), and FOXA1' rank is within top 10% changed genes. The resultant samples meeting this criterion were then plotted along the log transformed gene rank (*x* axis) and fold change of FOXA1 expression in cancers versus normal control (*y* axis). (B) Boxplot shows the differential expression of FOXA1 in rectal adenocarcinoma (The Cancer Genome Atlas), tongue squamous cell carcinoma [18], hepatocellular carcinoma [19], cutaneous follicular lymphoma and marginal zone B-cell lymphoma [20], melanoma [21]. (C) Scatterplot using the microarray data from a prostate cancer study [16] ($n = 30$) was shown with the log transformed expression level of FOXA1 (*x* axis) and CDKN2A (*y* axis). The data was fitted to a linear model with confidence interval (level of confidence interval is set to 0.95) showed in shaded gray area surrounding the smoothed straight line. (D) The samples ($n = 35$) from a prostate cancer study [17] were stratified into 4 groups according to their EZH2 levels (left). Pearson's correlation coefficient between FOXA1 and CDKN2A expression was separately calculated within the 4 groups (right). (E) The samples ($n = 204$) from a breast cancer study [15] were stratified into 20 groups according to their EZH2 levels (left). Pearson's correlation coefficient between FOXA1 and CDKN2A expression was separately calculated within the 20 groups (middle). The significance for Pearson's correlation was transformed ($-\log_{10}$) and shown (right). The dashed line in the right panel indicated a significance threshold of $p = 0.01$.

the repression of CDKN2A by PRC2 in senescence [3,6], Western blotting analysis indicated that sole depletion of EZH2 in LNCaP cell activated CDKN2A transcription in tumor settings (Fig. 2A). However, further removal of FOXA1 blunted CDKN2A reactivation, suggesting that FOXA1 is an essential activator for CDKN2A expression following the relief from PRC2 inhibition (Fig. 2A).

Although FOXA1-dependent expression change of CDKN2A was consistently observed in LNCaP cells, phenotypic examination was technically confounded by the heterogeneity nature of cancer cells, especially given the cooperation of FOXA1 and various pro-oncogenes in different cancer types [7,22,23]. To specify the significance of FOXA1 dependent regulation of CDKN2A, we generated a cell line stemmed from MCF-7 human breast adenocarcinoma cells, which bears genetically inactivated INK4 locus [24] but harbors a transgene (pSIR-tgCDKN2A) with minimal prerequisite set of regulatory elements for FOXA1-driven CDKN2A expression in senescence (Fig. S2) [6]. In agreement with our observations in senescence, the transgenic construct was epigenetically repressed by EZH2 after stable integration in MCF-7 cells, as the protein level of CDKN2A elevated following EZH2 knockdown (Fig. 2B). Importantly, further depletion of FOXA1 in a combinational transfection with both siRNA against EZH2 and FOXA1



	G1	S	G2
siNS	56.42%	26.22%	17.36%
siFOXA1	61.45%	15.58%	22.97%
siEZH2	72.84%	8.14%	19.02%
siEZH2/ siFOXA1	64%	15.18%	21.05%

Fig. 2. Loss of FOXA1 potentiates EZH2-mediated epigenetic repression of CDKN2A. (A) LNCaP cells were transfected with indicated double-stranded RNAi oligonucleotides. 48 h later, the cells were subjected to western blotting analysis with indicated antibodies. (B) Parental or transgenic MCF-7 cells stably integrated with CDKN2A cassettes in Fig. S2 were transfected with single siRNA against FOXA1 or EZH2, or a combination of the two. 48 h later, these cells were harvested for Western blotting analysis using indicated antibodies. GAPDH served as a loading control. (C) ChIP analysis of H3K4me3 and H3K27me3 enrichment on transgenic CDKN2A promoter in siRNA transfected cells as in (B). Data represent the mean + s.d. for triplicate experiments. (D) Parental or transgenic MCF-7 cells were transfected with siRNA as in (B). The growth curve of the cells was measured by MTT assays. (E) Transgenic MCF-7 cells stably integrated with CDKN2A cassettes was transfected with siRNA as in (B). The cells were first synchronized by serum starvation for 24 h and then cultured in medium containing FBS for 12 h. The cells were then collected for cell cycle analysis by flow cytometry. The flow cytometry data was also represented as bar plot and shown in the table.

greatly reduced such activation (Fig. 2B). Consistent with the expression changes, ChIP experiments suggested that single depletion of EZH2 caused more enrichment of active H3 lysine 4 trimethylation (H3K4me3) and less enrichment of EZH2-mediated H3K27me3 modification (Fig. 2C); while further depletion of FOXA1 suppressed this trend as indicated by decreased H3K4me3 enrichment on the transgenic CDKN2A promoter (Fig. 2C).

Phenotypic evidence supported the oncogenic functionality of EZH2 and FOXA1, as removal of either protein inhibits cancer cell proliferation in both parental and transgenic MCF-7 cells, albeit more pronounced in EZH2- than FOXA1-silenced cells (Fig. 2D). However, in the transgenic cells simultaneous knockdown of both proteins showed less growth arrest compared with the effect when single EZH2 was silenced; in contrast, in 9p21-deleted parental cells [24], double knockdown showed synergistic effects (Fig. 2D). This difference correlated with the inducible transgenic CDKN2A expression in the derived cells while CDKN2A is permanently silenced in the parental cells due to genetic deletion of INK4 locus (Fig. 2B). Cell cycle data in transgenic MCF-7 cells was consistent with MTT results, as less G1-phase cells and more

S-phase cells in double knockdown cells than them in EZH2-depleted cells (Fig. 2E). These phenotypic data also highlights the point that CDKN2A acts downstream of FOXA1 to regulate carcinogenesis.

3.3. Depletion of FOXA1 enables RAS- and EZH2-overexpression mediated oncogenic transformation

We then examined FOXA1's contribution to RAS induced oncogenic transformation where the CDKN2A-mediated senescence is one of major barriers against RAS-mediated tumorigenicity [25]. In one experimental setting, we overexpressed retroviral EZH2 together with RAS in its constitutive-activated form (H-RAS G12V) in normal human diploid fibroblast 2BS cells to stimulate their anchorage-independent growth, reasoning EZH2 would enhance this transformation process by inhibition of several tumor suppressors including CDKN2A [5]. However, the results indicated that ectopic expression of EZH2 was not sufficient to abrogate RAS-elicited senescence barrier, as judged by the negligible colony formation after 18-day culture (Fig. 3A). Western blotting analysis confirmed this point as significant level of CDKN2A survives in the presence of both RAS and EZH2 (Fig. 3B). However, consistent with above observations in cancer cells, simultaneous overexpression of EZH2 and RAS along with FOXA1 knockdown resulted in successful tumorigenic transformation of normal 2BS cells, indicated by the significant colony expansion on anchorage-independent culturing medium (Fig. 3A). This experiment was performed with two independent RNAi sequences against FOXA1, thus minimizing the RNAi-based off-target effect. Further Western blotting analysis confirmed that FOXA1 removal caused the complete silencing of CDKN2A (Fig. 3B). These data suggested that the everlasting FOXA1 proteins could be sufficient to counteract with overexpressed EZH2 and to impede carcinogenesis by maintaining a balanced expression of CDKN2A.

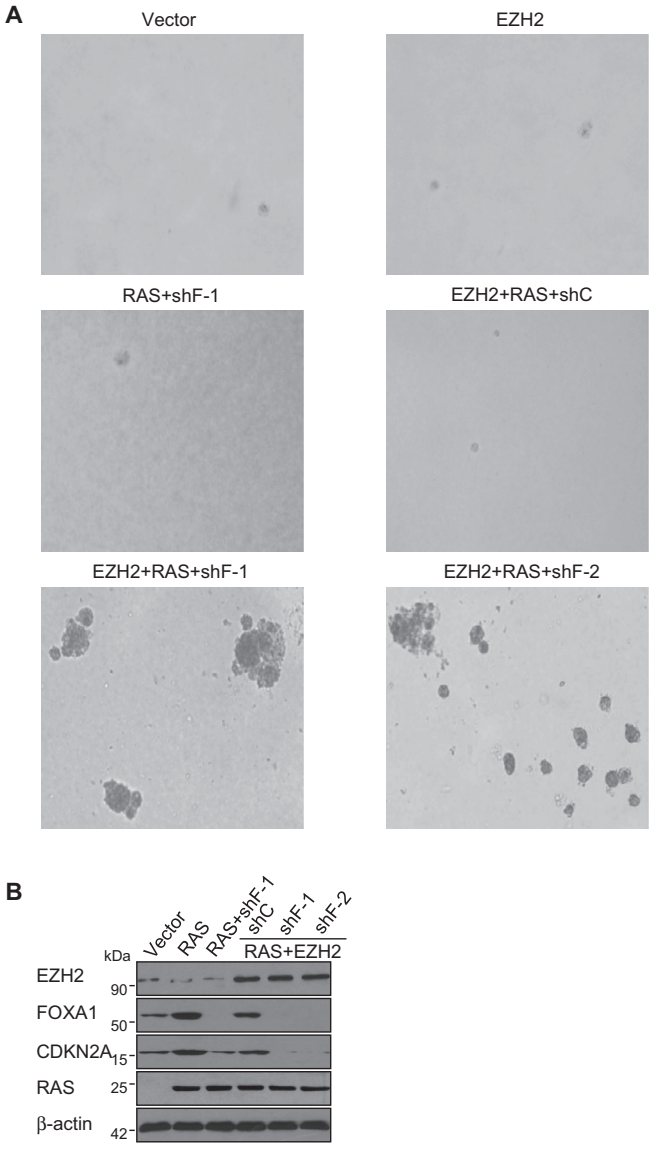


Fig. 3. Depletion of FOXA1 enables RAS- and EZH2-overexpression mediated oncogenic transformation. (A) 2BS cells were transduced with retroviral vector, or a combination of EZH2, H-RAS V12 (RAS), and two shRNAs against FOXA1 (shF-1 and shF-2), or a scramble control vector (shC). Anchorage-independent growth was determined by assaying colony formation in soft agar at an initial seeding density of ~5000 cells per well (six-well plate). After 18 days, the plates were photographed. (B) Western blotting with indicated antibodies was performed using cell lysates transduced as in (A) but on a regular plate to validate the infection efficiency.

3.4. Molecular basis for the antagonism between FOXA1 and EZH2 on transcriptional regulation

We noted that in transgenic MCF-7 cells, FOXA1 knockdown would lead to prominent H3K27me3 enrichment at transgenic CDKN2A promoter (Fig. 2C). This point together with the insufficiency of EZH2 overexpression to block RAS-induced CDKN2A activation in the presence of FOXA1, implied FOXA1 might directly regulates enzymatic activity of EZH2. To test this possibility, we set up an *in vitro* histone H3 trimethylation system, with purified core PRC2 component (EZH2, SUZ12, EED, AEBP and RbAp48) and oligonucleosome as its optimal substrate [26] (Fig. 4A). The methyl-transfer reaction was efficiently catalyzed by PRC2, however, inclusion of purified FOXA1 severely impaired this reaction (Fig. 4B). Interestingly, FOXA1-dependent enzymatic inhibition correlates well with its histone binding ability [9] (Fig. 4C), as C-terminal-depleted FOXA1 (ΔCt) failed to inhibit EZH2's HMTase activity (Fig. 4B). In contrast, the DNA binding domain-depleted FOXA1 (ΔDBD) is sufficient to bind histone and to inhibit EZH2's HMTase activity (Fig. 4B and C). Importantly, inclusion of synthesized C-terminal peptide essential for FOXA1' nucleosome binding ability [9] but not control peptide suppresses PRC2-catalyzed lysine 27 trimethylation as the full-length FOXA1 protein, implying the target sites of FOXA1 on core histone is crucial for PRC2-nucleosome reaction (Fig. 4D).

4. Discussion

Despite the understanding of FOXA1-mediated CDKN2A activation in senescence, it remains unclear on the deregulation of FOXA1/CDKN2A axis in cancer biology. This manuscript bridged the gap between FOXA1-mediated cellular senescence and its relevance for tumor development, which further supported the senescence barrier hypothesis [25]. Although the pro-oncogenic roles of FOXA1 have been demonstrated in specialized cancers, its broad implications in multiplex organogenesis implied more varied functional roles in carcinogenesis. Indeed, bioinformatics survey empowered by the comprehensive gene expression database Oncomine revealed a distinctive expression pattern of FOXA1 with significant down-regulation in brain, colorectal, gastric, liver, head and neck, lymphoma and pancreatic cancers (Fig. 1). In line with our observations, recent findings in endometrial cancer (EC) revealed that FOXA1 declined in EC compared with normal endometrium and forced expression of FOXA1 suppressed the progression of EC [27]. Indeed, loss of FOXA1 in prostate and breast cancer cells leads to CDKN2A inactivation in tumor settings (Fig. 2). Mechanistically, the antagonism between FOXA1 and

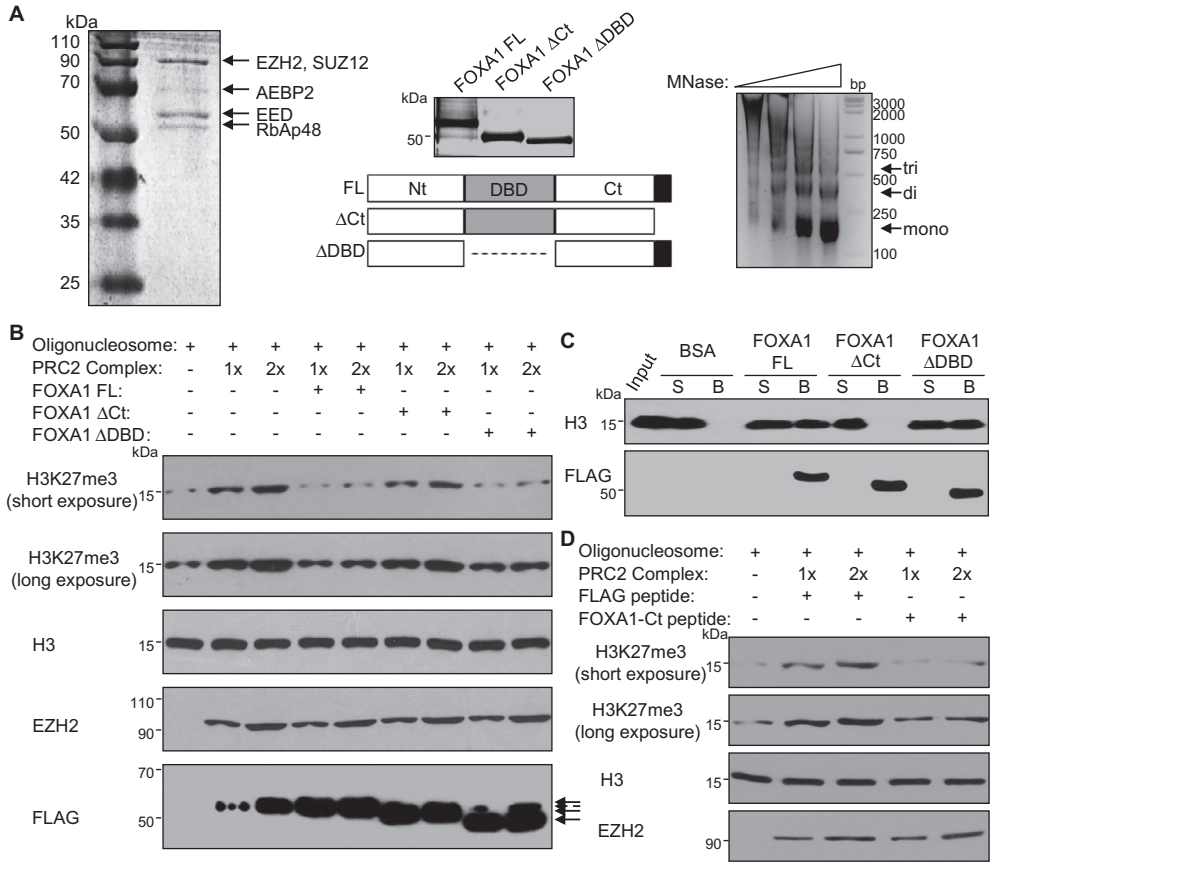


Fig. 4. Molecular basis for the antagonism between FOXA1 and EZH2 on transcriptional regulation. (A) Components of PRC2-catalyzed histone methylation reaction system. Coomassie Blue staining of purified PRC2 complex (EZH2, SUZ12, EED, AEBP2 and RbAp48) was shown in the left. Silver staining of purified FLAG-tagged full-length (FL), C-terminal-deleted (ΔCt), and DNA binding domain-deleted (ΔDBD) FOXA1 was shown in the middle. Micrococcal nuclease (MNase) digestion of reconstituted nucleosomes was resolved by 2% agarose gel and shown in the right. Partial MNase digestion generated a nucleosomal DNA ladder with visible mono-, di-, and trinucleosomal fragments indicated. (B) FOXA1 inhibits the enzymatic activity of PRC2. Oligonucleosomes purified from HeLa cells were incubated with PRC2 complex in the absence or presence of wild-type or deletion mutants of FOXA1. The reaction products were analyzed by Western blotting with indicated antibodies. The three arrows in the anti-FLAG panel indicated FLAG-tagged full-length and mutated FOXA1 proteins (FL, ΔCt, and ΔDBD). The dashed arrow indicated FLAG-tagged EED in the purified PRC2 complex. (C) Histone binding assay with wild-type or deletion mutants of FOXA1. Purified full-length or deletion mutants of FOXA1 proteins were immobilized on M2 beads and were further incubated with reconstituted nucleosome. The bound fractions (B) and supernatants (S) were subjected to Western blotting analysis with anti-H3 antibody. Bovine serum albumin (BSA) was used as negative control. (D) HMTase was performed as in (B). The use of control (FLAG) peptide or synthesized peptide from C-terminal portion of FOXA1 was indicated.

PRC2 was confirmed to be a key switch for oncogene elicited CDKN2A activation and senescence barrier in carcinogenesis, and FOXA1's opposing effect was attributable to its histone binding motif. This novel finding pinpoints the potential implication of FOXA1 in regulating histone modifications in addition to its known functions in physical reconfiguration of chromatin [9]. On the other hand, we noted that simple depletion of FOXA1 failed to bypass RAS-mediated senescence barrier, and this might be due to the residual CDKN2A expression without EZH2 overexpression (Fig. 3B). Furthermore, considering that FOXA1-mediated senescence was specifically dependent on CDKN2A [6], additional targets that FOXA1 does not regulate but EZH2 affects might contribute to EZH2-enhanced oncogenic transformation. Nevertheless, the requirement of FOXA1-depletion for sufficient CDKN2A inhibition in carcinogenesis and overall data supported a senescence-barrier function of FOXA1 as an activator of CDKN2A to counteract its transcriptional repression by PRC2 in the malignance process of cancers.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.092.

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