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FOXA1 mediates $p^{16INK4a}$ Activation during Cellular Senescence

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Anke Sparmann

1st Editorial Decision 05 July 2012

Thank you for submitting your research manuscript (EMBOJ-2012-82234) to our editorial office. It has now been seen by three referees whose comments to the authors you will find enclosed.

As you can see below, all referees appreciate the demonstration that FoxA1 promotes cellular senescence via the transcriptional activation of p16INK4a. However, the principal concern expressed by at least two reviewers is that the study remains too preliminary at this stage. In particular, the molecular mechanism by which FoxA1 modulates PRC function remains vague and requires further substantiation. Furthermore, the interesting aspect that the core region of the predicted FoxA1 binding motif at the distal interaction site in the p16 promoter carries a Diabetes-associated SNP is not sufficiently developed. Although we appreciate that a thorough analysis might be beyond the scope of the current manuscript, we nevertheless would like to stress that this potential link to Diabetes does add significantly to the general interest of the study. The issues mentioned above would have to be addressed by a considerable amount of additional work. However, since this appears feasible based on the constructive suggestions made by the referees, we would be willing to grant the opportunity to significantly extend and revise the current manuscript. As this will entail time-consuming experimentation, we would understand if you might decide to seek potential rapid publication elsewhere.

However, in case you embark on revisions for our general and strongly selective title, I urge you to take the specified demands into careful consideration to avoid disappointments later in the process. I

should add that it is our policy to allow only a single major round of revision and that it is therefore important to address all raised concerns at this stage. To give you this opportunity, I would be happy to extend our revision deadline to six month.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1

This manuscript explores the role of FoxA1 in senescence. The authors show that FoxA1 is the most up-regulated Forkhead factor during senescence and that FoxA1 directly binds to and regulates p16 expression. They show that FoxA1 is involved in histone positioning around the p16 regulatory element and that this involves PRC complexes. They also explore additional FoxA1 associated regions and investigate the genomic impact of a SNP that has been associated with diabetes. They authors claim that this SNP affects FoxA1 binding capacity.

Overall this is a very good manuscript on an important, but poorly understood process. The link between FoxA1 and p16 regulation and the impact on senescence is novel. The data are of high quality and the conclusions, for the most part, are correct. I did have a couple of minor concerns.

- The Western blot for FoxA1 in Figure 5B is not convincing. This needs to be repeated.
- My major concern is the work on the SNP. I'm not sure why this is included, but it doesn't add to the paper at all. In fact, the first 5 figures are solid and convincing, but figure 6 is confusing and not relevant. First of all, the SNP does not land in a FoxA1 motif. The DNA sequence with the SNP does not resemble a Forkhead motif and I even ran the sequence through motif prediction tools (using non stringent thresholds), but a Forkhead motif was never predicted by any approach I tested. As such, this region may be a genuine FoxA1 binding event, but it is not a direct FoxA1 associated region. As such, what the SNP does is not clear because it does not affect a FoxA1 motif.
- The link with diabetes SNP is confusing. This paper is about senescence and not diabetes. Although it is interesting that FoxA1-mediated regulation of p16 may be important for diabetes, this should constitute a completely different story. I would recommend that the entire figure 6 be removed from this paper.
- The discussion could use rewording. Some of the text (particularly the last two paragraphs) doesn't make sense.

Referee #2

This study identifies a novel function for the transcription factor FOXA1 in regulating induction of the p16INK4a tumor suppressor during replicative and oncogene-induced senescence. The authors have analyzed expression of a panel of Forkhead genes in human diploid 2BS fibroblasts at various passages and report that FOXA1 is significantly up-regulated in senescent cells and in cells undergoing oncogene-induced senescence (OIS), as well as tissues of older mice. FOXA1 induction in 2BS cells was required for replicative senescence and was shown to regulate expression of p16INK4a. A putative FOXA1 binding element was identified in the p16 promoter through ChIP, promoter-reporter, and in vitro binding experiments. FOXA1 binding resulted in eviction of a positioned nucleosome in the p16INK4a promoter, consistent with FOXA1's role as a pioneer transcription factor, and was associated with loss of polycomb group proteins and repressive chromatin marks. Finally, the authors identified a FOXA1 binding element located 150 kb upstream of the p16INK4a promoter. The putative binding site corresponds to a C to T polymorphism associated with type II diabetes, and they demonstrate increased affinity of the mutant site for

FOXA1. The authors propose that this polymorphism increases p16INK4a levels in pancreatic β cells, enhancing senescence and impairing insulin secretion.

The observation that FOXA1 activates p16INK4a and regulates senescence is a novel and important finding that identifies a new function for FOXA1. This work should be of interest to a wide audience. However, the paper contains a number of shortcomings that require significant revisions and additional experimental data. The link to diabetes is intriguing but lacks direct proof and, therefore, this part of the work should be de-emphasized (including removing the statement about diabetes in the Abstract) unless more supporting data are provided.

Major concerns:

1. In Fig. 2A, the authors show that ectopic FOXA1 expression induces growth arrest and senescence. It is critical to address whether these phenotypic features are dependent on up-regulation of p16INK4a by knocking down p16INK4a.

2. In Fig. 2B, it would be informative if the authors include a growth assay of the aged 2BS cells depleted for FOXA1. Does FOXA1 knockdown prevent cells from arresting?

3. Fig. 2D should include a FOXA1 knockdown experiment to investigate the requirement for FOXA1 in OIS. In this regard, it would be very interesting to test whether FOXA1 ablation promotes transformation in combination with other oncogenic factors, and to survey cancer genome and gene expression databases for inverse associations of FOXA1 with various cancers.

4. Fig. 3D. The EMSA would be more convincing if cell extracts were used to demonstrate endogenous FOXA1 binding to its putative site in the p16 promoter and the FOXA1 complex was verified using antibody supershift assays.

5. The authors should construct a point mutation in the putative FOXA1binding site in the p16INK4a promoter to determine if this eliminates FOXA1 responsiveness in their reporter assay.

6. Fig. 5D. The effect of siEZH2 on FOXA1 and p16INK4a levels is very modest and it is not clear if this supposed increase is significant. The mRNA levels for these two proteins should be analyzed, as this may provide a better quantitative measure of the effect of EZH2 depletion.

7. The putative FOXA1-dependent enhancer located -150 kb from the p16INK4a promoter was not characterized functionally. Since important regulatory sequences are often conserved evolutionarily, it would be informative to perform a phylogenetic comparison of the FOXA1 binding element and its surrounding sequences. In addition, the enhancer could be placed proximally to the p16INK4a promoter-reporter construct (or a heterologous promoter) to test whether it displays FOXA1-dependent enhancer activity.

8. In Fig. 6C, right panel, no FOXA1 binding to the SNP region is detected for the C/C (presumably "wild-type") genotype. However, this seems to contradict Fig. 6A, where binding to this region is induced in old 2BS cells. The authors should determine the SNP genotype of 2BS cells to determine if one or both alleles carry the T polymorphism, thus accounting for the strong binding of FOXA1.

9. The study would benefit from more extensive phenotypic characterization of the C-T SNP. Does FOXA1 over-expression in the lymphocyte cell lines cause growth arrest and senescence, and is the effect more pronounced in T/T cells?

10. p14ARF is another important senescence-associated gene that is expressed from the CDKN2A locus through a different promoter. The authors should determine whether FOXA1 regulates both transcripts, or selectively induces p16INK4a.

11. The paper is not well written, and the text needs extensive editing to correct English usage and grammar, replace inappropriate scientific wording, and improve clarity.

Minor concerns:

1. The fact that FOXA2 is also highly induced in senescent cells suggests the possibility of redundancy between these two factors. Have the authors investigated a similar function for FOXA2?

2. The analysis of FOXA1 deletion mutants shown in Fig. 3E has no clear purpose. In my opinion, these experiments do not add significantly to the conclusions of the study and should be removed.

3. The right panels of Fig. 4A are redundant with the bottom left panel of Fig. 4A. The right panels could be removed or moved to Supplementary Data. Also, it is recommended to clarify the labeling of the horizontal axis for Fig. 4D. What do the numbers 1-8 represent, and what region of the promoter is shown here?

4. The FOXA1 Western blot in Fig. 5B is of poor quality and should be replaced with a better blot.

Referee #3

General comments:

The paper by Li et al deals with the regulation of senescence and p16 by FoxA1. Senescence induction by FOXA1 and mechanisms proposed for FOXA1-induced p16 activation are of interest. However the work tackles many diverse points regarding the mechanism without demonstrating them in a solid manner. While the core observations presented in the manuscript (foxa1 playing a role in senescence by regulating p16) are solid, the rest of the manuscript (the relation with polycomb proteins, the possible effect on nucleosome repositioning, and the regulation via an enhancer region) are just touched very slightly. I believe that while the data shown in figures 1-3 and further detail in specific parts of the mechanism will make a very compelling story, the current paper is not in a shape ready to be published. A series of additional experiments would thus be needed to strengthen the conclusions, and a profound restructuring is also needed, excluding data that is interesting but very preliminary.

Specific comments:

- In Figure 1A, FOXA2 is up-regulated with replicative senescence together with FOXA1. FOXA1 and FOXA2 are close paralogs sharing a highly conserved DBD and cooperating in development. This would be good to mention in the results description. Do they have the same/distinct/cooperating functions in senescence and p16 regulation?. In addition, do the timing of induction of foxa1 and p16 coincide during serial passage?

- In Figure 2A, the proliferation curve of FOXA1 overexpressing cells need to be shown for more than 15 days. At this point it is not clear if the cells will stop or continue to proliferate. Furthermore, BrdU and SAHFs quantification should be included for a better description of the phenotype. Finally in the main text, references to this Figure are not coherent ("right upper panel" and "right lower panel") .

- Only one shRNA or siRNA is used in the experiments. The phenotype induced should be confirmed with another independent sequence for shRNA and siRNA.

- Related to Figure 2D: is shFOXA1 also affecting p16 level in the RAS system? This is the OIS system studied in Figure 1 and 4 rather than the BRAF system. In the right panel, the mRNA level of p16 shown should be relative to the level observed in the empty vector cells set at 1 or 100%.

- Is FOXA1-induced senescence overcome by shp16?

- In Figure 3E, the amount of each FOXA1 protein expressed (wt and mutants) needs to be shown by Western Blot for drawing conclusions from the luciferase data.

- In Figure 4C, the demonstration of FOXA1 effect on nucleosomes on the p16 promoter is not convincing. Which primers are used for ChIP? Effect of FOXA1 overexpression or knock down on both H1 and H3 ChIPs should be shown as well as on nucleosomal DNA enrichment as done in Figure 4A.

- In Figure 5, the effect of PRC2 knock down on FOXA1 level is not very convincing as the FOXA1 WB in shC/shSUZ12 cells in Figure 5B is not very clear and siEZH2 does not affect FOXA1 level in Figure 5D. Also, what are the ChIP primers used on FOXA1 promoter?.

-More importantly, in Fig 5D, the experiment were performed in MCF7 cells, that are reported to have a homozygous deletion at the cdkn2a locus, and to the best of my knowledge don't express a functional, full-length p16. This experiment should be performed in primary fibroblasts.

-How is FOXA1 suppose to modulate the action of PRC complexes? Is there a physical interaction between FOXA1 and any member of the PRC1 or PRC2 complexes?

- Although the possibility of FOXA1 modulating nucleosome location at the Ink4a promoter is exciting, it would need to be proved in detail that this happens during senescence and also that is directly dependent of FOXA1.

- Is FOXA1 binding to this 150 kb distal element modulating p16 level?

Minor comments

- IgG should be included in the ChIP presented in Figure 6C.

- In Figure 2B, additional read-outs such as proliferation curve, BrdU incorporation and colony formation assay should be included to prove the effect of knocking down FOXA1 on cellular senescence.

- In Figure 6B, what are the primers used for 3C?

- It would be good to propose a model for p16 regulation by FOXA1 to summarize the work and discuss it.

- The Materials and Methods section is very incomplete. Paragraphs describing the other reagents and techniques used have to be included there or in the Supplementary (plasmids, cell lines, tissue samples, primers, RNAi sequences, Western Blot, RT-PCR, ChIP, EMSA,...).

- There are some mistakes in the writing (grammar, word choice, sentence construction) which would need to be corrected to allow comfortable reading of this manuscript.

1st Revision - authors' response 28 December 2012

Response to Editors' comments-

1. EDITOR: *In particular, the molecular mechanism by which FoxA1 modulates PRC function remains vague and requires further substantiation.*

Authors: To substantiate our observation on the interplay of FOXA1 and PRC2 complex for transcriptional regulation of $p^{16INK4a}$, we performed series of experiments according to reviewers' comments as the followings:

1. We repeated the western blotting assay with improved RNAi effect against SUZ12 and the consequent up-regulation of FOXA1 and $p16^{INK4a}$ could be clearly observed (Figure 5B). In line with the results from loss-of-function experiment, overexpression of EZH2 in Ras-mediated OIS blunted the up-regulation of FOXA1 (Figure 5E).
2. To test the requirement of FOXA1 for PRC2-removal mediated $p16^{INK4a}$ activation, we co-transduced 2BS fibroblast cells with short hairpin RNAi (shRNA) to knock down FOXA1 and EZH2 simultaneously. Western blotting and real-time reverse-transcriptase PCR results supported that FOXA1 was indispensible for the action of PRC2 on $p16^{INK4a}$ because further

depletion of FOXA1 abolished the up-regulation of $p16^{INK4a}$ following EZH2 knock down (Figure 5D).

3. To get molecular insight of FOXA1's modulation of PRC2 function, we performed chromatin immunoprecipitation and western blotting experiments and observed mutually exclusive binding of FOXA1 and PRC2 at $p^{16INK4a}$ promoter. Concretely, enforced EZH2 overexpression weakened FOXA1-mediated $p16^{INK4a}$ up-regulation in OIS model (Figure 5E), while ectopic expression of FOXA1 promoted the dismissal of PRC2 complex from $p16^{INK4a}$ promoter (Figure 5F). On the other hand, depletion of EZH2 promoted association of FLAG-tagged FOXA1 with $p16^{INK4a}$ promoter (Figure 5G). Co-immuoprecipitation assay suggested FOXA1 and PRC1/2 did not interact physically (Supplementary Figure 4B) and thus their mutually exclusive interplay could be indirectly derived from their connection on histones (Figure 4). This point was reasonable, as previous research of FOXA1's recruitment on chromatin revealed that its presence was influenced by local epigenetic context in breast and prostate cancer (Lupien M, et.al., 2008, *Cell* **132:** 958-970).

Together with the finding that FOXA1 was transcriptional repressed by PRC2 on its promoter (Figure 5A-C), we hope these experiments could substantiate our point "FOXA1 was not only responding to depression of PRC2, but also implicated in reinforcing the active epigenetic remodeling at $p16^{INK4a}$ promoter to orchestrate a feedforward loop governed by PRC2-removal for fully activated $p16^{INK4a}$ transcription."

2. EDITOR: *Furthermore, the interesting aspect that the core region of the predicted FoxA1 binding motif at the distal interaction site in the p16 promoter carries a Diabetes-associated SNP is not sufficiently developed. Although we appreciate that a thorough analysis might be beyond the scope of the current manuscript, we nevertheless would like to stress that this potential link to Diabetes does add significantly to the general interest of the study.*

Authors: We appreciated the editor's point on this issue, and in the revised version we strengthened the functional link between rs10811661 DNA variant and FOXA1 regulated $p16^{INK4a}$ transcription.

The distal "T" containing allele was first proved to be a genuine functional enhancer through luciferase reporter assay, and the enhancer's activity was evidenced to be dependent on FOXA1 binding site at promoter region (Figure 6C). Importantly, one nucleotide substitution of "T" to "C" nearly abolished such activity, underscoring the significance of the DNA variant rs10811661 (Figure 6C). Furthermore, real-time reverse-transcriptase PCR assay indicated that ectopic expression of FOXA1 in GM10860 lymphocyte homozygous for rs10811661-T achieved greater $p16^{INK4a}$ expression than that of GM10851 lymphocyte homozygous for rs10811661-C (Figure 7C).

However, due to the transformed nature (inactivation of retinoblastoma protein) of these lymphocytes, they were not suitable for evaluation of $p16^{INK4a}$ controlled cell growth and senescence phenotypes. Alternatively, we created a transgenic construct hosted in a self-inactivating retroviral vector, which was composed of $p16^{INK4a}$ promoter, $p16^{INK4a}$ CDS, and the enhancer element carrying C- or T-allele (Figure 7D). With the removal of endogenous $p16^{INK4a}$ through RNAi, we observed enhanced cell cycle arrest and SA-β-galactosidase activity in 2BS fibroblast cells harboring T-allele construct, in coordination with its higher $p16^{INK4a}$ expression resulted from RNAi-resistant CDS of the exogenous transgene (Figure 7D). These data supported that the DNA variation at the -150kb away enhancer elements directly affected FOXA1 binding and further impacted $p16^{INK4a}$-centered senescence programs.

Regarding to the potentially association of rs10811661 and diabetes, it was a very good and significant point to directly evaluate the impact of rs10811661 DNA variant on the function of pancreatic b cell or other insulin response cells. However, non-transformed cells maintained in our lab was either heterozygous for T/C or were not fit for experiment due to the lack of control cells with paired allele. The best choice as generation of knock-in mouse based on this DNA variant was not available at this stage given by its consumption on the cost and timing. Thus, we hope the editors and reviewers will understand that due to reagent limitations, we are currently not able to fully address this very important point. And accordingly, we removed relative text in abstract section and toned down our statement on its potential implications in diabetes.

Response to reviewers' comments-

REFEREE #1

1. REFEREE #1: *The Western blot for FoxA1 in Figure 5B is not convincing. This needs to be repeated.*

Authors: We have repeated the western blotting assay, and the data has been replaced in the revised manuscript in Figure 5B.

2. REFEREE #1: *My major concern is the work on the SNP. I'm not sure why this is included, but it doesn't add to the paper at all. In fact, the first 5 figures are solid and convincing, but figure 6 is confusing and not relevant. First of all, the SNP does not land in a FoxA1 motif. The DNA sequence with the SNP does not resemble a Forkhead motif and I even ran the sequence through motif prediction tools (using non stringent thresholds), but a Forkhead motif was never predicted by any approach I tested. As such, this region may be a genuine FoxA1 binding event, but it is not a direct FoxA1 associated region. As such, what the SNP does is not clear because it does not affect a FoxA1 motif.*

Authors: We appreciated the reviewer's point. In the revised manuscript, we toned down our argumentation on the connection between rs10811661 SNP and diabetes in the abstract and result sections. Alternatively, we strengthened the functional link between this DNA variant and FOXA1 regulated $p16^{INK4a}$ transcription, due to the importance of enhancer binding capacity of Forkhead family factors for gene transcription (Zaret KS, Carroll JS, 2011, Genes & development 25: 2227-2241). The luciferase reporter assay and functional evaluation of DNA variant at rs10811661 supported the association between "T" allele enhancer element and FOXA1 dependent senescence progression (Figure 6C and Figure 7).

The motif information in original manuscript was not demonstrated clearly. The FOXA1 binding motif was retrieved from jaspar database (http://jaspar.genereg.net) with default threshold and a detailed illustration of the FOXA1 binding motif and relevant SNP information was provided in Supplementary Figure 6. Furthermore, both EMSA (Figure 7A) and luciferase assay (Figure 6C) supported the predicted DNA sequence was a genuine FOXA1 binding motif. We hope these data helpful to clarify this point.

3. REFEREE #1: *The link with diabetes SNP is confusing. This paper is about senescence and not diabetes. Although it is interesting that FoxA1-mediated regulation of p16 may be important for diabetes, this should constitute a completely different story. I would recommend that the entire figure 6 be removed from this paper.*

Authors: We agree with the reviewer's point on the SNP's link with diabetes. As stated above, we modified relevant text in revised manuscript. And we also hope the data in Figure 6 and 7 would consolidate our central conclusion on FOXA1 dependent regulation for $p16^{INK4a}$ transcription.

4. REFEREE #1: *The discussion could use rewording. Some of the text (particularly the last two paragraphs) doesn't make sense.*

Authors: Agree with the reviewer. The discussion was rewritten with rewording part removed.

REFEREE #2

1. REFEREE #2: *The link to diabetes is intriguing but lacks direct proof and, therefore, this part of the work should be de-emphasized (including removing the statement about diabetes in the Abstract) unless more supporting data are provided.*

Authors: We agree with the reviewer's point. The relative text has been de-emphasized and the statement in the abstract section was deleted.

Major concerns:

2. REFEREE #2: *In Fig. 2A, the authors show that ectopic FOXA1 expression induces growth arrest and senescence. It is critical to address whether these phenotypic features are dependent on up-regulation of p16INK4a by knocking down p16INK4a.*

Authors: We appreciate the reviewer's point. An epistasis analysis was performed by co-expression of FOXA1 with a shRNA against $p16^{INK4a}$ or a control vector in 2BS cells. Growth arrest and senescence measured by DNA synthesis and SAHF formation indicated that FOXA1 induced senescence was greatly reduced when $p16^{INK4a}$ was further removed (Figure 2E). This experiment implied the dependency of FOXA1 on $p16^{INK4a}$ to initiate senescence.

3. REFEREE #2: *In Fig. 2B, it would be informative if the authors include a growth assay of the aged 2BS cells depleted for FOXA1. Does FOXA1 knockdown prevent cells from arresting?*

Authors: In the revised manuscript, we transduced middle-aged 2BS cells with two independent lentiviral shRNA against FOXA1 or a non-silence control. Growth curve together with SAHF formation, BrdU incorporation and colony formation assays of 2BS cells were examined following FOXA1 depletion (Figure 2B). Elevated BrdU incorporation, augmented colony formation, decreased presence of SAHF and continuous cell proliferation could be observed following FOXA1 knockdown, suggesting that FOXA1 knockdown prevent 2BS cells from growth arresting.

4. REFEREE #2: *Fig. 2D should include a FOXA1 knockdown experiment to investigate the requirement for FOXA1 in OIS. In this regard, it would be very interesting to test whether FOXA1 ablation promotes transformation in combination with other oncogenic factors, and to survey cancer genome and gene expression databases for inverse associations of FOXA1 with various cancers.*

Authors: We appreciate the reviewer's point. To better demonstrate the requirement for FOXA1 in OIS, we silenced its expression in Ras or BRAF V600E-induced 2BS cells with shRNA (Figure 2D and Supplementary Figure 1B). Consistent with decline of $p16^{INK4a}$ expression, phenotypic changes as elevated BrdU incorporation and less presence of SAHF were observed in OIS following FOXA1 ablation, indicative of reduced senescence barrier (Figure 2D).

Overexpression of Ras and EZH2 accompanied by FOXA1 depletion greatly promoted colony formation in soft agar relative to control RNAi (Supplementary Data for reviewer only). In concert with the in vitro transformation experiment, cancer gene expression profile analysis revealed the inverse association between FOXA1 and neoplastic progression in several cancer types, such as gastric adenocarcinoma, desmoplastic medulloblastoma, pancreatic ductal adenocarcinoma, and colon adenocarcinoma (Supplementary Figure 7). Detailed relationship between Ras, EZH2, FOXA1 and $p16^{INK4a}$ during tumorigenesis deserves a specific investigation and this project is ongoing in our lab.

5. REFEREE #2: *Fig. 3D. The EMSA would be more convincing if cell extracts were used to demonstrate endogenous FOXA1 binding to its putative site in the p16 promoter and the FOXA1 complex was verified using antibody supershift assays.*

Authors: We performed EMSA with endogenous nuclear lysate from young or aged 2BS cell (Supplementary Figure 2A). In line with the expression of FOXA1 in aged cells, a shift could be observed when aged but not young nuclear lysate was used. The specificity as a FOXA1 binding motif was confirmed by competition assay with unlabeled wild-type or mutated probes. Furthermore, super-shift caused by FOXA1 antibody but not by normal IgG was also evidenced (Supplementary Figure 2A).

6. REFEREE #2: *The authors should construct a point mutation in the putative FOXA1binding site in the p16INK4a promoter to determine if this eliminates FOXA1 responsiveness in their reporter assay.*

Authors: A point mutation reporter was constructed by substitution of the four most conserved thymine bases for cytosine (Figure 3D). And this mutant completely lost its responsiveness to FOXA1 in the luciferase report assay.

7. REFEREE #2: *Fig. 5D. The effect of siEZH2 on FOXA1 and p16INK4a levels is very modest and it is not clear if this supposed increase is significant. The mRNA levels for these two proteins should be analyzed, as this may provide a better quantitative measure of the effect of EZH2 depletion.*

Authors: We repeated western blotting analysis with efficient knockdown of EZH2 in 2BS cells. The new data in Figure 5D demonstrated significant up-regulated protein levels of FOXA1 and $p16^{INK4a}$ responding to EZH2 knockdown. The mRNA analysis also supported the transcriptional up-regulation $p16^{INK4a}$ correlated with EZH2 decline (Figure 5D).

8. REFEREE #2: *The putative FOXA1-dependent enhancer located -150 kb from the p16INK4a promoter was not characterized functionally. Since important regulatory sequences are often conserved evolutionarily, it would be informative to perform a phylogenetic comparison of the FOXA1 binding element and its surrounding sequences. In addition, the enhancer could be placed proximally to the p16INK4a promoter-reporter construct (or a heterologous promoter) to test whether it displays FOXA1-dependent enhancer activity.*

Authors: We appreciate the reviewer's point on this issue, and in the revised version we strengthened the functional link between rs10811661 DNA variant and FOXA1 regulated $p16^{INK4a}$ transcription through several independent approaches.

Luciferase reporter assay confirmed that the distal "T" containing allele was a genuine functional enhancer, and such activity was dependent on FOXA1 binding site at promoter region (Figure 6C). Importantly, one nucleotide substitution of "T" to "C" nearly abolished the enhancer activity, underscoring the significance of the DNA variant rs10811661 (Figure 6C). In addition, real-time reverse-transcriptase PCR indicated that ectopic expression of FOXA1 in GM10860 lymphocyte homozygous for rs10811661-T achieved greater $p16^{INK4a}$ expression than that of GM10851 lymphocyte homozygous for rs10811661-C (Figure 7C). These data supported that the DNA variation at the -150kb away enhancer elements directly affected FOXA1 binding and further impacted FOXA1-mediated transcriptional activation of $p16^{INK4a}$.

Sequence conservation analysis and phylogenetic comparison of the FOXA1 binding element and its surrounding sequences was performed and illustrated in Supplementary Figure 5. The core region of predicted FOXA1 binding motif maps to sites of evolutionary conservation among several mammals, implying its biological relevance.

The enhancer/promoter (heterologous promoter) reporter was constructed and measured as stated above (Figure 6C).

9. REFEREE #2: *In Fig. 6C, right panel, no FOXA1 binding to the SNP region is detected for the C/C (presumably "wild-type") genotype. However, this seems to contradict Fig. 6A, where binding to this region is induced in old 2BS cells. The authors should determine the SNP genotype of 2BS cells to determine if one or both alleles carry the T polymorphism, thus accounting for the strong binding of FOXA1.*

Authors: We appreciate the reviewer's point. Genotyping of 2BS cells revealed its heterozygous status (C/T) at rs10811661. Interestingly, sequencing results of FOXA1 bound DNA fragments in ChIP experiments suggested their inclination for T allele as the ratio of T-allele clones versus C-allele clones was 8:2. This information was added to the text.

10. REFEREE #2: *The study would benefit from more extensive phenotypic characterization of the C-T SNP. Does FOXA1 over-expression in the lymphocyte cell lines cause growth arrest and senescence, and is the effect more pronounced in T/T cells?*

Authors: We appreciate the reviewer's point. However, due to the transformed nature (inactivation of retinoblastoma protein) of these lymphocytes, they were not suitable for phenotypic characterization of the C-T SNP. Alternatively, we created a transgene construct hosted in a self-inactivating retroviral vector, which was composed of $p16^{INK4a}$ promoter, $p16^{INK4a}$ CDS, and the enhancer element carrying C- or T-allele (Figure 7D). With the removal of endogenous $p16^{INK4a}$ through RNAi, we observed enhanced cell cycle arrest and SA-β-galactosidase activity in 2BS fibroblast cells harboring T-allele transgenic construct, in coordination with its higher $p16^{INK4a}$ expression resulted from RNAi-resistant CDS of the exogenous transgene (Figure 7D).

With reference to other phenotypic characterization, especially the aging of pancreatic b cell, we quoted our discussion in the response to EDITOR's comment, "Regarding to the potentially association of rs10811661 and diabetes, it was a very good and significant point to directly evaluate the impact of rs10811661 DNA variant on the function of pancreatic b cell or other insulin response cells. However, non-transformed cells maintained in our lab was either heterozygous for T/C or were not fit for experiment due to the lack of control cells with paired allele. The best choice as

generation of knock-in mouse based on this DNA variant was not available at this stage given by its consumption on the cost and timing. Thus, we hope the editors and reviewers will understand that due to reagent limitations, we are currently not able to fully address this very important point. And accordingly, we removed relative text in abstract section and toned down our statement on its potential implications in diabetes."

11. REFEREE #2: *p14ARF is another important senescence-associated gene that is expressed from the CDKN2A locus through a different promoter. The authors should determine whether FOXA1 regulates both transcripts, or selectively induces p16INK4a.*

Authors: $p14^{ARF}$ expression did not change with ectopic FOXA1 expression as determined by real-time reverse-transcriptase PCR (Figure 2C). The selective induction of $p16^{INK4a}$ but not $p14^{ARF}$ was consistent with the specific recruitment of FOXA1 on $p16^{INK4a}$ promoter but not on $p14^{ARF}$ promoter (Figure 3A).

12. REFEREE #2: *The paper is not well written, and the text needs extensive editing to correct English usage and grammar, replace inappropriate scientific wording, and improve clarity.*

Authors: Majority of this paper has been rewritten and we hope these changes would improve the English usage in the revised manuscript.

Minor concerns:

13. REFEREE #2: *The fact that FOXA2 is also highly induced in senescent cells suggests the possibility of redundancy between these two factors. Have the authors investigated a similar function for FOXA2?*

Authors: We agree with the reviewer. FOXA2 is up-regulated in replicative senescence, and the relevant information was added in the result section. Actually, in our preliminary test, FOXA1 but not FOXA2 could drive the up-regulation of $p16^{INK4a}$, and this result was provided in the Supplementary Figure 1A. To better clarify this point, the following text was added in the discussion section: "FOXA2 as a close paralog of FOXA1 was similarly identified to be up-regulated in replicative senescence (Figure 1A), however, enforced expression of FOXA1 but not FOXA2 drove the induction of $p16^{INK4a}$ in 2BS fibroblast cells consistently (Supplementary Figure 1A). Although the molecular basis for this differential regulation was currently unknown and correlation of FOXA2 and $p16^{INK4a}$ in other biological context was not determined in this study, distinct sets of target genes of these paralogs were recently demonstrated in the adult liver through genome-wide location analysis and such functional diversification was proposed to confer the maintenance of both genes during evolution (Bochkis et al, 2012, PLoS genetics 8: e1002770)".

14. REFEREE #2: *The analysis of FOXA1 deletion mutants shown in Fig. 3E has no clear purpose. In my opinion, these experiments do not add significantly to the conclusions of the study and should be removed.*

Authors: We hope the luciferase reporter assay with FOXA1 truncated mutants in Figure 3E could consolidate the importance of sequence-specific association between FOXA1 and $p16^{INK4a}$ promoter. To clarify this point, the following text "In addition, luciferase activity analysis with domain mutagenesis of FOXA1 indicated that DNA-binding domain (DBD) was essential for $p16^{INK4a}$ promoter activation (Supplementary Figure 2B), reinforcing the importance of sequence-specific association between FOXA1 and $p16^{INK4a}$ promoter." was added in the result section. And this figure has been moved to Supplementary data due to its relative lower significance.

15. REFEREE #2: *The right panels of Fig. 4A are redundant with the bottom left panel of Fig. 4A. The right panels could be removed or moved to Supplementary Data. Also, it is recommended to clarify the labeling of the horizontal axis for Fig. 4D. What do the numbers 1-8 represent, and what region of the promoter is shown here?*

Authors: The right panels of original Figure 4A have been removed. Original Figure 4D was moved to Supplementary data due to stronger evidence given in Figure 4 in the revised version. And a

schematic illustration for the primer sets used in original Figure 4D has been added in the top panel of substitute Supplementary Figure 3A.

16. REFEREE #2: *The FOXA1 Western blot in Fig. 5B is of poor quality and should be replaced with a better blot.*

Authors: We have repeated the western blotting assay, and the data has been added in the revised manuscript in Figure 5B.

REFEREE #3

1. REFEREE #3: *In Figure 1A, FOXA2 is up-regulated with replicative senescence together with FOXA1. FOXA1 and FOXA2 are close paralogs sharing a highly conserved DBD and cooperating in development. This would be good to mention in the results description. Do they have the same/distinct/cooperating functions in senescence and p16 regulation?. In addition, do the timing of induction of foxa1 and p16 coincide during serial passage?*

Author: We agree with the reviewer. FOXA2 is up-regulated in replicative senescence, and the relevant information was added in the result section. Actually, in our preliminary test, FOXA1 but not FOXA2 could drive the up-regulation of $p16^{INK4a}$, and this result was provided in the Supplementary Figure 1A. To better clarify this point, the following text was added in the discussion section: "FOXA2 as a close paralog of FOXA1 was similarly identified to be up-regulated in replicative senescence (Figure 1A), however, enforced expression of FOXA1 but not FOXA2 drove the induction of $p16^{INK4a}$ in 2BS fibroblast cells consistently (Supplementary Figure 1A). Although the molecular basis for this differential regulation was currently unknown and correlation of FOXA2 and $p16^{INK4a}$ in other biological context was not determined in this study, distinct sets of target genes of these paralogs were recently demonstrated in the adult liver through genome-wide location analysis and such functional diversification was proposed to confer the maintenance of both genes during evolution (*Bochkis et al, 2012, PLoS genetics* 8: e1002770)". The expression of $p16^{INK4a}$ was included in the heatmap, and the result suggested that both FOXA1 and $p16^{INK4a}$ were induced at very late passage in 2BS cells (Figure 1A).

2. REFEREE #3: *In Figure 2A, the proliferation curve of FOXA1 overexpressing cells need to be shown for more than 15 days. At this point it is not clear if the cells will stop or continue to proliferate. Furthermore, BrdU and SAHFs quantification should be included for a better description of the phenotype. Finally in the main text, references to this Figure are not coherent ("right upper panel" and "right lower panel").*

Author: We appreciated the reviewer's comment. In the revised manuscript, the population-doubling analysis in FOXA1 overexpressing 2BS cells was performed for ~40 days, and the results in Figure 2A indicated severe growth retardation in cells overexpressing FOXA1, which ceased to proliferate ~20 days post infection. Quantitative analysis of BrdU and SAHFs was carried out and the resulted indicated ectopic expression of FOXA1 resulted in decreased BrdU incorporation and elevated presence of SAHF (Figure 2A). And the references to Figure 2A in the main text have been corrected.

3. REFEREE #3: *Only one shRNA or siRNA is used in the experiments. The phenotype induced should be confirmed with another independent sequence for shRNA and siRNA.*

Author: The phenotype changes with FOXA1 depletion were examined with two independent RNAi in the revised manuscript and the new data has been added in Figure 2.

4. REFEREE #3: *Related to Figure 2D: is shFOXA1 also affecting p16 level in the RAS system? This is the OIS system studied in Figure 1 and 4 rather than the BRAF system. In the right panel, the mRNA level of p16 shown should be relative to the level observed in the empty vector cells set at 1 or 100%.*

Author: We analyzed $p16^{INK4a}$ level and phenotypic changes in Ras induced OIS in 2BS cells with two independent shRNA against FOXA1. The results suggested shFOXA1 severely impaired Ras induced $p16^{INK4a}$ expression and phenotypic features of senescence (Figure 2D). The mRNA level of $p16^{INK4a}$ in BRAF systems was shown relative to the level observed in the empty vector cells set at 1 as suggested by the reviewer (Supplementary 1B).

5. REFEREE #3: *Is FOXA1-induced senescence overcome by shp16?*

Author: We appreciate the reviewer's point. An epistasis analysis was performed by co-expression of FOXA1 with a shRNA against $p16^{INK4a}$ or a control vector in 2BS cells. Growth arrest and senescence measured by DNA synthesis and SAHF formation indicated that FOXA1 induced senescence was greatly reduced when $p16^{INK4a}$ was further removed (Figure 2E). This experiment implied FOXA1-induced senescence was overcome by $p16^{INK4a}$ knockdown.

6. REFEREE #3: *In Figure 3E, the amount of each FOXA1 protein expressed (wt and mutants) needs to be shown by Western Blot for drawing conclusions from the luciferase data.*

Author: Expression of wild-type and truncated FOXA1 was examined through western blotting assay, and the data has been added in Supplementary Figure 2B.

7. REFEREE #3: *In Figure 4C, the demonstration of FOXA1 effect on nucleosomes on the p16 promoter is not convincing. Which primers are used for ChIP? Effect of FOXA1 overexpression or knock down on both H1 and H3 ChIPs should be shown as well as on nucleosomal DNA enrichment as done in Figure 4A.*

Author: This comment was tightly linked with comment 11.ChIP experiment in original Figure 4C was performed with primer 6 as in Figure 4A to demonstrate the effect of FOXA1 on nucleosomes at the $p16^{INK4a}$ promoter. However, due to the low resolution of sonication ChIP (250-400bp), realtime PCR with other primers listed in Figure 4A detected comparable H3 and H1 binding as the one with primer 6 at $p16^{INK4a}$ promoter (data for reviewer only). To better illustrate the removal of -2-positioning nucleosome was caused by FOXA1, we ectopically expressed FOXA1 in young 2BS cells and depleted endogenous FOXA1 in middle aged 2BS cells and then performed high resolution MNase mapping assay to measure histone positioning changes with same sets of primers in Figure 4A. The results in Figure 4C, suggested the sufficiency and necessity of FOXA1 for the -2-positioning nucleosome movement specifically, which was recapitulating such changes during replicative senescence as shown in Figure 4A.

Although ChIP experiment is not suitable for precise measurement of histone positioning, it is useful to demonstrate chromatin dynamics in a wider range and also useful to argue against the possibility that chromatin structure changes in senescent fibroblast might be derived from increased accessibility of the DNA for MNase digestion. Hence, two control loci -1kb or +1kb away from TSS were analyzed in the revised manuscript and the results implied chromatin steadiness in these control loci contrary to -2-positioning nucleosome regardless of FOXA1 expression (Figure 4D and Supplementary Figure 3B).

These experiments strengthened our conclusion that FOXA1 promotes nucleosome loss at $p16^{INK4a}$ promoter.

8. REFEREE #3: *In Figure 5, the effect of PRC2 knock down on FOXA1 level is not very convincing as the FOXA1 WB in shC/shSUZ12 cells in Figure 5B is not very clear and siEZH2 does not affect FOXA1 level in Figure 5D. Also, what are the ChIP primers used on FOXA1 promoter?.*

Author: We repeated the western blotting assay with improved RNAi effect against SUZ12 or EZH2 in 2BS cells and the up-regulated protein level of FOXA1 and $p16^{INK4a}$ could be clearly observed (Figure 5B and 5D). ChIP primers used to detect binding of PRC2 on the four regions (region a, b, c, d) of FOXA1 promoter were illustrated in Figure 5C. The primer to amplify the MIPOL1 promoter served as negative control.

9. REFEREE #3: *More importantly, in Fig 5D, the experiment were performed in MCF7 cells, that are reported to have a homozygous deletion at the cdkn2a locus, and to the best of my knowledge don't express a functional, full-length p16. This experiment should be performed in primary fibroblasts.*

Author: The experiment was performed in 2BS fibroblast cells as suggested by the reviewer. Depletion of FOXA1 with either of two independent shRNA abolished the up-regulated $p16^{INK4a}$ following EZH2 knockdown suggested FOXA1 was required for EZH2 silence-mediated $p16^{INK4a}$ up-regulation in primary fibroblasts (Figure 5D).

10. REFEREE #3: *How is FOXA1 suppose to modulate the action of PRC complexes? Is there a physical interaction between FOXA1 and any member of the PRC1 or PRC2 complexes?*

Author: To substantiate our observation on the interplay of FOXA1 and PRC2 complex for transcriptional regulation of $p^{16INK4a}$, we performed series of experiments as the followings:

1. We repeated the western blotting assay with improved RNAi effect against SUZ12 and the consequent up-regulation of FOXA1 and $p16^{INK4a}$ could be clearly observed (Figure 5B). In line with the results from loss-of-function experiment, overexpression of EZH2 in Ras-mediated OIS blunted up-regulation of FOXA1 (Figure 5E).
2. To test the requirement of FOXA1 for PRC2-removal mediated $p^{16INK4a}$ activation, we co-transduced 2BS fibroblast cells with short hairpin RNAi (shRNA) to knock down FOXA1 and EZH2 simultaneously. Western blotting and real-time reverse-transcriptase PCR results supported that FOXA1 was indispensible for the action of PRC2 on $p16^{INK4a}$ because further depletion of FOXA1 abolished the up-regulation of $p16^{INK4a}$ following EZH2 knock down (Figure 5D).
3. To get molecular insight of FOXA1's modulation of PRC2 function, we performed chromatin immunoprecipitation and western blotting experiments and observed mutually exclusive binding of FOXA1 and PRC2 at $p16^{INK4a}$ promoter. Concretely, enforced EZH2 overexpression weakened FOXA1-mediated $p16^{INK4a}$ up-regulation in OIS model (Figure 5E), while ectopic expression of FOXA1 promoted the dismissal of PRC2 complex from $p16^{INK4a}$ promoter (Figure 5F). On the other hand, depletion of EZH2 promoted association of FLAG-tagged FOXA1 with $p16^{INK4a}$ promoter (Figure 5G).

We performed co-immuoprecipitation assay with FOXA1 and representative PRC1/2 components, and the result suggested FOXA1 and PRC1/2 did not interact physically (Supplementary Figure S3D) and thus their mutually exclusive interplay more likely was indirectly derived from their connection on histones (Figure 4 and Supplementary Figure 4B). This point was reasonable, as previous research of FOXA1's recruitment on chromatin revealed that its presence was influenced by local epigenetic context in breast and prostate cancer (Lupien M, et.al., 2008, *Cell* **132:** 958-970).

11. REFEREE #3: *Although the possibility of FOXA1 modulating nucleosome location at the Ink4a promoter is exciting, it would need to be proved in detail that this happens during senescence and also that is directly dependent of FOXA1.*

Author: As stated in our response to comment #7, the nucleosomal dynamics at $p16^{INK4a}$ promoter during senescence and its dependency on FOXA1 were tested through two independent approaches: an in vivo realtime PCR based assay to map nucleosomal DNA from MNase digested mononucleosomes with high-resolution and a conventional ChIP assay with histone antibodies to directly measure their spatial pattern on $p16^{INK4a}$ promoter. Both overexpression and knockdown of FOXA1 supported its sufficiency and necessity for -2-positioning nucleosome movement, consistent with nucleosome location changes during replicative senescence (Figure 4). These experiments consolidated our conclusion as specific nucleosome movement was restricted around FOXA1 binding sites (Figure 4A).

12. REFEREE #3: *Is FOXA1 binding to this 150 kb distal element modulating p16 level?*

Author: In the revised manuscript, we utilized several independent approaches to test the functional impact of this 150 kb distal element modulating $p16^{INK4a}$ expression. First, the distal "T" containing allele was proved to be a genuine functional enhancer through luciferase reporter assay, and the enhancer's activity was evidenced to be dependent on FOXA1 binding site at promoter region (Figure 6C).

Second, one nucleotide substitution of "T" to "C" nearly abolished such activity, underscoring the significance of the DNA variant rs10811661 (Figure 6C). Furthermore, RT-qPCR results indicated

that ectopic expression of FOXA1 in GM10860 lymphocyte homozygous for rs10811661-T achieved greater $p16^{INK4a}$ expression than that of GM10851 lymphocyte homozygous for rs10811661-C (Figure 7C).

Third, we created a transgene construct hosted in a self-inactivating retroviral vector, which was composed of $p16^{INK4a}$ promoter, $p16^{INK4a}$ CDS, and the enhancer element carrying C- or T-allele (Figure 7D). With the removal of endogenous $p16^{INK4a}$ through RNAi, we observed enhanced cell cycle arrest and SA-β-galactosidase activity in 2BS fibroblast cells harboring T-allele construct, in coordination with its higher $p16^{INK4a}$ expression resulted from RNAi-resistant CDS of the exogenous transgene (Figure 7D).

These data supported that the DNA variation at the -150kb away enhancer elements directly affected FOXA1 binding and further impacted $p16^{INK4a}$-centered senescence programs.

Minor comments

13. REFEREE #3: *IgG should be included in the ChIP presented in Figure 6C.*

Author: We included the ChIP results with normal IgG as shown Figure 7B (original Figure 6C) in the revised manuscript.

14. REFEREE #3: *In Figure 2B, additional read-outs such as proliferation curve, BrdU incorporation and colony formation assay should be included to prove the effect of knocking down FOXA1 on cellular senescence.*

Author: As suggested by the reviewer, additional read-out experiments including proliferation curve, BrdU incorporation and colony formation assay were all performed and these data were added in Figure 2B in the revised manuscript.

REFEREE #3: *In Figure 6B, what are the primers used for 3C?*

Author: 3C analysis in Figure 6B was used to examine the ligation products generated by re-ligation of Xba I processed DNA fragments from ‘‘constant fragment’’ and “candidate interacting fragment”. The primers in Figure 6B were used to amplify the re-ligated products composed of $p16^{INK4a}$ promoter as the “constant fragment’’ and DNA stretches -150kb or -70kb away from TSS as “candidate interacting fragment”. This information was added in the relevant figure legend and the detailed sequences for pro--150kb and pro--70kb were included in Materials and methods section.

15. REFEREE #3: *It would be good to propose a model for p16 regulation by FOXA1 to summarize the work and discuss it.*

Author: We appreciate the reviewer’s comment. The model for transcriptional regulation of $p16^{INK4a}$ by FOXA1 examined in the current study has been demonstrated in Figure 7E, and relevant description has been added in the discussion section.

16. REFEREE #3: *The Materials and Methods section is very incomplete. Paragraphs describing the other reagents and techniques used have to be included there or in the Supplementary (plasmids, cell lines, tissue samples, primers, RNAi sequences, Western Blot, RT-PCR, ChIP, EMSA,...).*

Author: The Materials and Methods were complemented in the revised manuscript with detailed description of reagents and techniques used in this study provided.

17. REFEREE #3: *There are some mistakes in the writing (grammar, word choice, sentence construction) which would need to be corrected to allow comfortable reading of this manuscript.*

Author: Majority of this paper has been rewritten and we hope these changes would improve the English usage in the revised manuscript.

2nd Editorial Decision 23 January 2013

Thank you for submitting your revised manuscript for our consideration.

It has now been seen once more by two of the original referees, whose comments are provided below. The reviewers acknowledge that their major concerns have been addressed, and they both are in general supportive of publication in The EMBO Journal. Nevertheless, referee #2 suggests a few minor changes that should be implemented.

In addition, could you please indicate the number of biological replicates the data presented in Figure 2A, 2B and 2E is based upon. If possible, add error bars to indicate the standard deviation. In general, please be sure to clarify in all Figure legends the number of biological replicates as well as the statistical test used to gage significance.

Finally, we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I am taking this opportunity to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

I will now return your manuscript to you for one additional round of minor revision. After that we should be able to proceed with formal acceptance and production of the manuscript!

If you have any questions, please do not hesitate to contact me directly.

REFEREE COMMENTS

Referee #2

The authors have made a number of significant revisions to the previous manuscript and have addressed the major criticisms of this referee. A few minor changes will improve the current version.

1. The EMSA shown in Supplementary Figure 2A using nuclear lysates from 2BS cells demonstrates that endogenous FOXA1 binds to the site in the proximal p16Ink4A promoter. This experiment is superior to the one shown in Figure 3C, and it is therefore recommended that the authors switch the two experiments.

2. As noted by referee #1, the SNP in the distal enhancer is not in a central part of the FOXA1 recognition sequence, at least as indicated by the sequence logo shown. Can the authors cite previous work indicating that this part of the element is critical for binding of forkhead proteins? (It is acknowledged that Figure 7A shows that the "C" allele has reduced affinity for FOXA1 relative to the "T" allele.)

3. The text still contains several grammatical errors and poor sentence construction in places. The writing should be corrected before publication of the manuscript.

Referee #3

The authors have addressed most of the comments of this and other reviewers. The manuscript has improved substantially and although there is still not a clear link between FoXA2 and some of the proposed mechanisms of regulation of the INK4a/ARF locus, I am satisfied with the revision.

2nd Revision - authors' response 29 January 2013

Response to reviewers' comments-

REFEREE #2

1. REFEREE #2: *The EMSA shown in Supplementary Figure 2A using nuclear lysates from 2BS cells demonstrates that endogenous FOXA1 binds to the site in the proximal p16Ink4A promoter. This experiment is superior to the one shown in Figure 3C, and it is therefore recommended that the authors switch the two experiments.*

Authors: EMSA results in Figure 3C and Supplementary Figure 2A were switched as suggested by the reviewer.

2. REFEREE #2: *As noted by referee #1, the SNP in the distal enhancer is not in a central part of the FOXA1 recognition sequence, at least as indicated by the sequence logo shown. Can the authors cite previous work indicating that this part of the element is critical for binding of forkhead proteins? (It is acknowledged that Figure 7A shows that the "C" allele has reduced affinity for FOXA1 relative to the "T" allele.)*

Authors: We appreciate the reviewer's point. A recent publication on breast cancer risk–associated SNPs revealed rs4784227-T variant allele at the same position (position 8) as rs10811661 in FOXA1 binding motif could favor its recruitment over rs4784227-C allele (Cowper-Sal lari, et.al., 2012, Nature genetics 44: 1191-1198), and this reference was added to strengthen our conclusion.

3. REFEREE #2: *The text still contains several grammatical errors and poor sentence construction in places. The writing should be corrected before publication of the manuscript.*

Authors: Grammatical errors and improper constructed sentences were corrected and the text was rechecked and modified for several rounds to improve the writing.

