molecular systems biology

Genome wide analysis of FOXO3 mediated transcription regulation through RNA polymerase II profiling

Astrid Eijkelenboom, Michal Mokry, Elzo de Wit, Lydia M Smits, Paulien E Polderman, Miranda H van Triest, Ruben van Boxtel, Almut Schulze, Wouter de Laat, Edwin Cuppen, Boudewijn M T Burgering

Corresponding author: Boudewijn Burgering, University Medical Center Utrecht

Review timeline:

Submission date:	23 February 2012
Editorial Decision:	02 April 2012
Appeal:	17 April 2012
Editorial Decision:	20 April 2012
Re-submission:	10 October 2012
Editorial Decision:	30 October 2012
Revision received:	13 November 2012
Accepted:	10 December 2012

Editors: Andrew Hufton / Thomas Lemberger

Transaction Report:

(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.)

1st Editorial Decision 02 April 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees raise substantial concerns on your work, which, I am afraid to say, preclude its publication.

In general, the reviewers acknowledged that the data collected in this work could potentially provide some new insights into FOXO3 associated transcriptional regulation. Nonetheless, the reviewers clearly felt that this work, at present, was not yet sufficiently conclusive, and required substantial additional experimental evidence and analysis to rigorously support the main biological conclusions. Of particular note, the reviewers had concerns regarding the FOXO3-ER fusion protein and its ability to reproduce physiological FOXO3 regulation patterns. The second reviewer was initially somewhat more positive, but during the cross-commenting period s/he expressed clear support for the concerns raised by the other reviewers. The first reviewer also commented additionally on his/her last point, writing that some biological replication of the large-scale datasets would be crucial to assess the robustness of these conclusions.

Given the extensive nature of the additional experiments requested by these reviewers, and since some of their concerns seem to raise doubts about the direct physiological relevance of the results, we feel that we have no choice but to return this work with the message that we cannot offer to

publish it.

Nevertheless, the reviewers expressed interest in the topic of this work, and they do make constructive suggestions for further experiments. As such, we would like to suggest that we may be willing to reconsider an expanded submission based on this work. The additional experimental work requested by the reviewers is clearly substantial, and would require, at minimum, biological replication of at least some of the large-scale datasets (reviewer #1 felt that the FOXO3-ER ChIP-seq would be particularly crucial), and direct support for the ability of the FOXO3-ER fusion to mimic wt regulatory activity.

Any new submission would have a new number and receipt date, and we can give no guarantee about its eventual acceptability. If you do decide to follow this course then it would be helpful to enclose with your re-submission an account of how the work has been altered in response to the points raised in the present review.

I am sorry that the review of your work did not result in a more favorable outcome on this occasion, but I hope that you will not be discouraged from sending your work to Molecular Systems Biology in the future.

Thank you for the opportunity to examine this work.

_____

Reviewer #1 (Remarks to the Author):

Review on MSB-12-3601 ´ FOXO3 regulates gene expression from distal enhancers [a], Eijkelenboom et al.

In this manuscript, Eijkelenboom, Burgering and colleagues describe the genome-wide distribution of a tamoxifen-inducible FOXO3-ER fusion protein in human colon cancer cells. FOXO3-ER binding site are shown to be mainly intergenic and evolutionarily conserved, although a significant faction of sites are found within genes. To relate this distribution with transcriptional regulation, the authors compare FOXO3-ER Chip-seq data with RNA polymerase II distribution mapped by ChIP-seq with an antibody recognizing RBP1. Interestingly, FOXO3-ER binding sites show a mild elevation in levels of RBP1 in the presence of tamoxifen, suggesting that FOXO3-ER is a direct activator of transcription. A relationship between genes down-regulated by FOXO3a and FOXO3-ER binding was less obvious and this observation led to the assumption that these genes are not direct FOXO3 targets. Finally, through 4C-seq, the authors demonstrate that FOXO3-ER associates with pre-existing chromatin loops, suggesting that binding of FOXO3 to its target sites does not regulate loop formation but rather RNA Pol II engagement.

These data provide new insights into FOXO3 associated transcriptional regulation processes, and especially on the implication of FOXO3 in the modulation of activity of chromatin loops involving enhancer-promoter interactions.

Specific comments :

1. As mentioned in the discussion (page 15), about one third of FOXO3-ER binding sites are located within transcribed regions but the authors suggest that identification of these sites could be due to a nonspecific increase in background signal over transcribed regions. One way to help distinguish between genuine binding and nonspecific background could be to analyze H3K4me1 enrichment at a number of selected intragenic binding sites. Intragenic enhancers are not uncommon and could represent a significant fraction of FOXO3 binding regions. Thus a positive signal in H3K4me1 ChIP-qPCR would add biological relevance to the observed FOXO3 binding at intragenic sites.

2. It is clear from the presented data that FOXO3-ER binds pre-existing chromatin loops already loaded with RNA Pol II. This observation argues against a pioneering function of FOXO3 at enhancers. Thus, it is questionable whether FOXO3 has a genuine function at these sites. The conclusion drawn here is that engagement of FOXO3 stimulates Pol II recruitement as suggested by variation in RBP1 signal. Although this might be a correct interpretation of the data, the FOXO3-ER

regulated fraction of RBP1 loading is quite mild. This mild effect on Pol II engagement could however be associated with a stimulated production of eRNAs from the FOXO3-bound enhancers. Experiments could be carried out to analyze production of eRNAs upon tamoxifen addition. As mentioned by the authors, the mild effect of FOXO3-ER binding could be due to the involvement of other FOX proteins binding at the same sites. Thus, the authors should provide information on expression levels of other FOX proteins in DL23 cells and verify if the most expressed FOX proteins are enriched at selected FOXO3-ER binding sites.

3. Enrichment in motifs for GATA factors is found in FOXO binding regions and the authors hypothesise that GATA proteins could be pioneers for FOXO3. Proving this would greatly strengthen this manuscript. Knock-down of GATA factors expressed in DL23 cells followed by 3C and RBP1 ChIP-qPCR at selected sites (+/- TAM) could provide information on the putative implication of GATA factors in FOXO3 function.

4. A major drawback of this work concerns the use of a FOXO3-ER fusion protein to study the function of FOXO3. Although this fusion provides a very good system to control the nuclear translocation of FOXO3, it uses a part (LBD) of a transcription factor (ER) which is known to interact both with co-repressors and co-activators depending on cellular and chromatin contexts. Hence, the ER-LBD moiety of the fusion protein could recruit either co-repressors (and thus mask FOXO3-driven activity on chromatin) or co-activators which could in turn be responsible for the observed increase in Pol II signal. In order to be able to conclude that FOXO3 itself regulates RNA Pol II loading at its binding sites, the authors should provide evidence for the neutral effect of the ER-LBD moiety in the system used.

5. It is not clear from the Materials and Methods section how many biological replicates were subjected to the ChIP-seq procedure. Have ChiP-seq data been obtained with chromatin from a single experiment with a single biological sample in each condition or from chromatin extracted from a number of biological replicates and pooled ? This should be clarified.

Reviewer #2 (Remarks to the Author):

This is a novel and incisive study that will be of broad general interest to people working on both control of transcription and foxos and the diverse traits that they mediate. Particular strengths of the study are the quick, experimental activation of foxo3a in cells followed by rapid (4h and 24h) measurement of the changes in genome-wide binding not just of foxo3a itself but also of RNA polymerase II, together with changes in genome-wde patterns of RNA expression assessed by deep sequencing. In addition, the study provides new information on the way that foxo3a acts from enhancers to alter gene expression, through histone modifications and chromatin loops. These features make this work a major advance on previous work and it should be of wide interest to the readers of MSB. Such comments as I have are more to do with presentation than substance.

The title of the paper has to convey a message, but I am not sure that the current version has the balance quite right. Only about a third of the enhancers that the authors find to be regulated by foxo3a are distal to the genes that they regulate, although of course this finding is an interesting one.

It should be pointed out somewhere in the MS that the power of the induction system used comes with some downsides. Any cell-based study will have limitations in terms of the information gained about what happens in vivo in specific tissues, and the version of foxo3a used in the study was necessarily highly modified, with a tamoixfen-activated ligand-binding domain of the ER and a triple mutation to make it constitutively nuclear when activated. And who knows what other secondary modifications of the protein and co-factors are/are not present in this particular cell system with this particular version of the TF. As the authors point out in the Introduction to the paper, the mode of action of foxos is highly context-specific. These limitations probably mean that the data on the molecular mode of action of the TF are particularly robust and informative while the precise gene targets identified are less so. Of course it is reassuring to note that many of the targets identified are ones that would be expected from in vivo work and from the known functional outputs of the TF in vivo.

Introduction

There is an Oncogene review specifically on the role of foxos in ageing from Partridge and Bruening.

Results

The induction system needs a much more detailed description - I had to go to earlier publications to understand exactly what was being induced and how.

Pol II occupancy was monitored only for annotated genes, in one sense this negates the power of sequencing, which is that it can detect unannotated transcripts. I assume the main reason this was done was that transcript changes were monitored with microarrays rather than with sequencing?

Figure 1D It is not clear what the colour codes mean and the diagram needs more explanation in the figure legend.

Figure 2B legend 'ration' should be 'ratio'. In Figure 2A the 4h and 24h induction data going towards the 3' end of the genes look progressively more different - is there some sort of genuine time course of binding here?

The sentence starting ' Interestingly, the subset of distal FOXO3 binding regions.....' refers to the data in Figure 3C which gives an average phastcons score. This needs explanation - exactly what has been measured here to make the deduction of 'conservation' of distal binding sites?

Discussion

It is pointed out that pol II occupancy does not increase at motifs for TFs whose binding motifs are present in foxo3a bound regions. Could they be acting as co-repressors?

In the sentence starting 'GATA has been described to function.....' there is a 'for' missing before 'chromatin'

M and M

Heading should be: Quantification OF RNAPII occupancy

Recommendation

Accept with minor revision

Reviewer #3 (Remarks to the Author):

Manuscript by Eijkelenboom et al.
The work here presents a case for a relationship between FOXO3-mediated gene activation and RNA polymerase II (RNAPII) occupancy using ChIP-seq of both FOXO3 and RNAPII. The binding candidates showed enrichment for several known transcription factor sequence motifs. A substantial number of the candidate FOXO3 peaks were distal from their associated target gene and the authors demonstrate a concomitant increase in RNAPII occupancy, as well as H3K27ac enrichment (a widely reported marker of enhancer activity). Finally, the authors report 4C data suggesting a novel "spatial organization" between FOXO3 target genes and distal enhancers via chromatin looping. In short, the general interest is sufficient, the methodology is reasonable, but the results are not sufficiently convincing.

1)In general, peak-calling algorithms depend on arbitrary thresholds such as p-value or FDR cutoffs. Can authors go into more detail on how parameters were chosen to minimize false positives? Were input/mock controls used? Specifically, are the 9932 FOXO3 candidate-binding sites of high confidence, particularly in light of your observation that "not all regions fulfill the peak calling criteria"?

2)How are the fig1A densities calculated? What are you units for the y-axes? Are these binned read

counts, base pair counts?

3)In Figure 3A, is there any evidence of motif regulatory modules comprised of the enriched motifs? Since, FOX consensus motifs are present in only 45% of the peaks, do you have some intuition as to whether the remaining peaks are false positives or perhaps some alternate motif or sub-optimal FOX motif?

4)In Figure 4B, the distribution of the changes in RNAPII occupancy in some of the bins do not look normally distributed (long tail towards positive change). Perhaps a Kolmogorov-Smirnov test would be more appropriate?

5)For figures 5A and 5D, it also appears there are some non-normally distributed bins. Can a normality test be performed, or a K-S test in lieu of the unpaired t-test?

6)In the 2nd to last sentence on page 16, is there a typo at "...shown to be transiently required chromatin unfolding...."

Appeal 17 April 2012

First of all we thank you for the review of our manuscript. In your accompanying letter you indicate that you would be willing to consider a revised version of our manuscript. You also indicate two issues that you would like us to at least address satisfactorily. As we would like to work on resubmitting to MSB, I was hoping you could be somewhat more specific on these two issues, since this will hopefully avoid misunderstanding.

First, we have employed a widely used ER-system to induce FOXO3 activity in a ligand-independent manner. The advantage being that we can regulate a transcription factor, in our case FOXO3, without additional potentially confounding cell signaling. However, with the potential disadvantage as indicated by reviewer 1 of introducing ER effects that may/or may not interfere with FOXO3 effects. Thus the question is raised to show that the ER is neutral in our system (reviewer 1). We agree that the question is valid, however, and unfortunately, reviewer 1 does not provide a suggestion as to what he/she would consider an informative experiment in this respect.
Although we have ideas as to how we could approach this question, we also realize that none of these approaches provide definitive proof. One central issue in this respect is that it is impossible to activate endogenous FOXO3 without affecting other pathways. Both PI3K inhibitor LY and AKT inhibitor VIII, commonly used to activate endogenous FOXOs, will influence other signaling as well. For example AKT is known to regulate NcoR transcription repressor complex and thus will also not be neutral. Alternatively, we could analyse transcriptional regulation by a FOXO-DNA binding domain (DBD) fused to ER. In this case the ER-only will be targeted to the same FOXO sites and possible transcriptional regulation by the ER could be revealed (or not). However, others and we have shown that the DBD domain efficiently functions as a dominant-negative for endogenous FOXO and thus we can only reveal possible positive regulation by the LBD of the ER.

In order to prevent that we perform a substantial amount of additional experiments that may, from our perspective, but not necessarily from the reviewers perspective, provide some indication that indeed ER is neutral, we specifically ask for some guidance here. Otherwise this may result in a non-productive and even frustrating discussion arguing whether or not our additional work indeed sufficiently shows ER to be neutral. We realize that the burden of proof is on our side, but we do hope that you can sympathise with the underlying problem as to how to Osatisfy¹ the reviewer when possibilities are numerous but none are definite.
Finally with respect to the ER issue, we note that numerous previously reported FOXO target genes are also regulated by FOXO3-ER. FOXO3-ER also induces the same cellular responses as reported for endogenous FOXO including regulation of anti-oxidants, cell cycle progression etc.

Analysis of FOXO orthologs in C.elegans and Drosophila has revealed FOXO to likely act as a transcriptional activator only, confirming our results. Thus in all respects FOXO3a-ER thus far has shown to faithfully mimic endogenous FOXO. This at least corroborates the notion that ER is likely neutral.

Second, with respect to a replicate of the FOXO3a-ER Chip-Seq, we have performed several controls that validate the results of this Osingle¹ experiment. First, Q-PCRs in Figure 5E, S2, S5C consistently show enrichment of FOXO3 binding in all FOXO3 bound regions tested and endogenous FOXO3 binds to the same locations following LY treatment (Figure S2C). In addition, independent results on intergenic RNA pol II binding reveals a very significant overlap with FOXO3 binding sites (Figure 5, S5) and increases specifically in FOXO3 bound RNA pol II sites only. De novo motif analysis revealed Forkhead motif presence in increased intergenic RNA pol II peaks as well, in line with FOXO3
DNA binding in these regions. Although one may argue how many of the binding sites need to be independently confirmed, we believe that since these independent controls and genome wide datasets on RNA pol II are all in agreement with our initial results, no further confirmation is necessary. Nevertheless the FOXO3-ER Chip-seq can be repeated, although we believe for the reasons outlined above it is unlikely to be really informative.

We hope that with your insights on the issues outlined above we can come to a more rapid and hopefully positive outcome of the reviewing process. Especially, since we notice that within the field this study is highly appreciated as illustrated by the first author being selected to present these data on the recent Keystone ³eukaryotic transcription² meeting (Snowbird, Utah).

2nd Editorial Decision 20 April 2012

Thank you sending us your letter requesting additional clarification on our recent decision on your work (MSB-12-3601).

I have now had time read and consider the points you raise in letter. I fully recognize that addressing the issues raised by the reviewers may require substantial additional work, beyond what we would normally ask for a major revision. Indeed, this was the reason that we felt the need to reject this work, but suggested that a resubmission may be reconsidered in the future if you are interested in pursuing these additional experiments.

The first key issue was a concern by the reviewers that the ER fusion could introduce non-FOXO3 regulatory effects. The experiment you propose, testing a fusion of the ER LBD and the FOXO3 DBD, does seem like it could be potentially helpful. I recognize that this experiment would not be able to rigorously identify all potential ER-driven regulatory events (since the FOXO3 DBD may still have repressive activities), but it should be able to broadly support or reject the idea that the primarily activating activity of the ER-FOXO3 construct is driven by the FOXO3 domain and not the ER domain. If similar genomic patterns of transcriptional activation at FOXO3 binding sites are still observed, this would of course be troubling, and would probably require further investigation. I recognize that this experiment would likely require both ChIP-seq and RNA Pol seq experiments with this construct, which is far from trivial.

Secondly, the first reviewer was troubled by the lack of replication for the genomic datasets. This was a point that I discussed with the reviewer before rendering our previous decision, and the reviewer did indicate that this issue made it difficult to determine the broad reliability/reproducibility of these results. I recognize that you do conduct some validating small-scale ChIP experiments, but this validation does remain somewhat focused. It was also not entirely clear to me whether these tests were all derived from independent biological samples (presumably the LY experiments at least were independent samples). Clearly, additional validation of some kind would be needed with any resubmitted work, and repeating some of the main large-scale seq datasets may be the most direct way to accomplish this.

The gold standard here would be probably be repeating the FOXO3 ChIP-seq and the RNA PolII seq experiments on independent biological samples, which would then allow you to filter noisy genes or binding events out of subsequent analyses. This is not entirely trivial since genes with noisy expression might have functional or regulatory biases that could influence the subsequent results (e.g. Munsky et al, 2012). Nonetheless, repeating these experiments is clearly resource intensive, and I cannot rule out that you might be able to address this issue with a wider range of targeted validation experiments. For example, you could perhaps test RNA PolII and FOXO3 ChIP patterns for a selection of genes with different observed patterns of FOXO3 promoter binding and transcriptional response (binding & activation, binding & repression, no-binding & activation) on independent biological samples. This may not provide a global assessment of biological variability, but it might help to support the general reproducibility of the activation vs repression patterns.

I hope this additional information is useful as you plan your future experiments. As you probably understand, any decision on a resubmitted work will be handled in combination with advice from the expert reviewers, and I cannot guarantee a positive outcome at this stage.

Re-submission 10 October 2012

Please find enclosed our revised manuscript, which we would like to resubmit for publication in Molecular Systems Biology. Based upon the reviewers' comments we have revised our manuscript accordingly and addressed all issues raised.

The reviewers felt that our manuscript was not sufficiently conclusive for two main reasons. First, there was concern whether the FOXO3A3-ER fusion is able to reproduce physiological FOXO3 regulation and second the reviewers wished to see some more biological replicates. Following, our email exchange in which we expressed our ideas as to how to approach these questions and your answer to these suggestions we have addressed these issues accordingly.
In short, we have generated stable cell lines expressing a fusion protein of the DNA binding domain and nuclear localization signal (NLS) of FOXO3 to the ER moiety. Compared to the original FOXO3A3-ER, this construct lacks all transcriptional transactivation/regulatory domains of FOXO3. With these cell lines we could show that following 4OHT induction, indeed this fusion protein also binds to the same DNA sequences as the full length FOXO3A3-ER protein, but lacks all further effects (i.e. changes in histone acetylation, increased RNApolII occupancy at genes and enhancers, increased mRNA etc.). From this we conclude that the ER moiety is unlikely to be a confounding factor in our system.
Second, to address the issue of FOXO3A3-ER reproducing endogenous FOXO3 further, we have performed endogenous FOXO3 and RNApolII ChIP-seq following endogenous FOXO3 activation by treatment of cells with the PKB inhibitor VIII. In short the results show that FOXO3A3-ER is a faithful mimic of endogenous FOXO3, with the advantage that the signal to noise ratio in most experiments is better compared to endogenous FOXO3. Endogenous FOXO3 regulation is only possible in a context in which signalling towards FOXO3 is manipulated, hence the use of PKB inhibition here. However, inhibition of PKB will have impact on other transcription regulatory systems other than FOXO, which can be cooperative, inhibitory etc. and thus likely will result in more noise into the system. Actually, these results not only confirm that FOXO3A3-ER system faithfully reproduces endogenous FOXO3, but also that our rationale to start our experiments by using this 'ligand-independent' activation system was correct. These results also provide the requested biological replicates.

Below you will find a detailed response to all concerns/issues raised by the reviewers and we hope that with these additional experiments and changes our manuscript is now suited for publication.

Reviewer #1 (Remarks to the Author):

Specific comments :

1. As mentioned in the discussion (page 15), about one third of FOXO3-ER binding sites are located within transcribed regions but the authors suggest that identification of these sites could be due to a nonspecific increase in background signal over transcribed regions. One way to help distinguish between genuine binding and nonspecific background could be to analyze H3K4me1 enrichment at a number of selected intragenic binding sites. Intragenic enhancers are not uncommon and could represent a significant fraction of FOXO3 binding regions. Thus a positive signal in H3K4me1 ChIP-qPCR would add biological relevance to the observed FOXO3 binding at intragenic sites.

Three regions (Figure 6A, S7) we checked for H3K4me1 occupancy are within introns, where we also observe induction in histone acetylation, providing biological relevance for FOXO3 binding at these intragenic sites. Indeed a significant fraction of FOXO3 binding regions lies within genes. However, our analysis focuses on FOXO3 mediated changes in RNAPII occupancy at enhancers. For this we will have to exclude RNAPII signal from transcribed regions as changes in expression of the surrounding gene will confound the analysis. Therefore we focus on intergenic FOXO3 bound regions for further analysis.

2. It is clear from the presented data that FOXO3-ER binds pre-existing chromatin loops already loaded with RNA Pol II. This observation argues against a pioneering function of FOXO3 at enhancers. Thus, it is questionable whether FOXO3 has a genuine function at these sites. The conclusion drawn here is that engagement of FOXO3 stimulates Pol II recruitement as suggested by variation in RBP1 signal. Although this might be a correct interpretation of the data, the FOXO3-ER regulated fraction of RBP1 loading is quite mild.

To more clearly show the increase of RNAPII at intergenic regions, we have included graphs representing the average signal of the heat maps (Figure 5C).
Also from the supplementary figure 6D, showing three different enhancers, it becomes clear that there are differences in the level of RNAPII induction between FOXO3 bound regions, but the increase can be quite prominent. Moreover, the fold induction changes of RBP1 signal are at a similar range as changes of RBP1 occupancy over the gene bodies and changes in mRNA levels of regulated genes.

This mild effect on Pol II engagement could however be associated with a stimulated production of eRNAs from the FOXO3-bound enhancers. Experiments could be carried out to analyze production of eRNAs upon tamoxifen addition.

As initial attempts to measure eRNAs with Q-PCR based methods failed, we discussed this issue with Ramin Shiekhattar from the Wistar Institute, Philadelphia. In his experience, due to the short half-life, it is very difficult to observe eRNAs with Q-PCR and advised us to perform RNA-sequencing. As we are interested to analyze FOXO induced changes in production of eRNAs, we will pursue RNA-sequencing. This is however outside of the scope of this study, as this to our opinion than also requires further validation of existence and biological function of these eRNAs, and such an analysis will be published elsewhere.

As mentioned by the authors, the mild effect of FOXO3-ER binding could be due to the involvement of other FOX proteins binding at the same sites. Thus, the authors should provide information on expression levels of other FOX proteins in DL23 cells and verify if the most expressed FOX proteins are enriched at selected FOXO3-ER binding sites.

There are over 45 Forkhead box genes in the human genome. Likely not all will be expressed sufficiently in our cell system. Also, availability of antibodies of sufficient grade to all these FOX proteins is unlikely. Yet, even if we could show for one or more of these FOX proteins to bind by ChIP experiments, this will not address this issue. We would therefore need functional data, for example depleting specific FOX proteins and analyzing the effects on RNAPII occupancy, before we may conclude whether indeed this Forkhead is responsible for pre-existing RNAPII signal at enhancers. In case of these data being negative we have to continue and work our way to all the other 45 FOX proteins for finding another FOX protein for which we may even have to generate novel antisera etc. Although a valid question, we feel that this is at present clearly outside the scope of this study.

3. Enrichment in motifs for GATA factors is found in FOXO binding regions and the authors

hypothesise that GATA proteins could be pioneers for FOXO3. Proving this would greatly strengthen this manuscript. Knock-down of GATA factors expressed in DL23 cells followed by 3C and RBP1 ChIP-qPCR at selected sites (+/- TAM) could provide information on the putative implication of GATA factors in FOXO3 function.

This is indeed as the previous one a good suggestion. Exactly for this reason we therefore also discuss this in the discussion section, already indicating that for us this is a follow-up issue that we will definitely pursue. But as for testing all FOX proteins, we consider finding a pioneer factor for FOXO outside the scope of our current study and we are planning to pursue this for a follow-up study. For the initial conclusions of this study, these experiments in our opinion not necessary.

4. A major drawback of this work concerns the use of a FOXO3-ER fusion protein to study the function of FOXO3. Although this fusion provides a very good system to control the nuclear translocation of FOXO3, it uses a part (LBD) of a transcription factor (ER) which is known to interact both with co-repressors and co-activators depending on cellular and chromatin contexts. Hence, the ER-LBD moiety of the fusion protein could recruit either co-repressors (and thus mask FOXO3-driven activity on chromatin) or co-activators which could in turn be responsible for the observed increase in Pol II signal. In order to be able to conclude that FOXO3 itself regulates RNA Pol II loading at its binding sites, the authors should provide evidence for the neutral effect of the ER-LBD moiety in the system used.

To exclude any influence from the ER-LBD moiety, we have fused the FOXO3 DNA binding domain and nuclear localization signal to the ER-LBD moiety. This fusion still binds to Forkhead DNA binding domain, but lacks other FOXO3 domains important in for transactivation. We could not observe any effect from this fusion protein on FOXO3 target gene expression or RNAPII occupancy and histone acetylation at enhancers (Figures S7-S11). We can not completely rule out that the ER tag in some cases might specifically affect the FOXO3 response. Therefore we studied the activation of endogenous FOXO. In the first manuscript, we already provide some evidence that FOXO3A3-ER functions as endogenous FOXO. First target genes and processes fit with previously reported effects for FOXO activation. Second, the correlation between FOXO DNA binding and transcriptional activation has been reported for FOXO orthologs in C.elegans and Drosophila as well. In addition, we find FOXO3 binds to the same regions, as validated by Q-PCR to six regions (Figure S7). We have now performed additional experiments to show more elaborately that endogenous FOXOs, activated through PKB inhibition, can induce similar effects. More specifically, we show:

- Fig. S7: Fox six peaks we can repeatedly find binding of endogenous FOXO3 upon PI3K inhibition; the DBD fusion also binds to these regions (Figure S7A). In addition, PKB inhibition induces H3K27Ac levels at these same peaks, while no changes are observed upon activation of the DBD fusion (Figure S7B).
- Fig. S11: For six genes we can repeatedly find induction by both PKB and PI3K inhibition, similar to induction by FOXO3A3-ER, while activating the DBD only does not affect target gene expression.
- Fig. S10: RNAPII ChIP-seq was performed and overall changes in RNAPII occupancy of target genes is similar comparing PKB inhibition with activation of FOXO3A3-ER through 4OHT.
- Fig. S8: also ChIP-seq for endogenous FOXO3 and DBD fusion were performed. We can generally find an increased signal of both endogenous FOXO3 and DBD at the same regions where FOXO3A3-ER was bound.
- Fig. S9: at intergenic sites we see the same, both endogenous FOXO3 and DBD bind to these regions. While PKB inhibition also induces RNAPII around FOXO3 bound intergenic regions, we could not find any change upon activation of DBD-ER.

Since the DBD shows the ER tag generally does not induce any changes on intergenic regions and PKB inhibition induces endogenous FOXO3 to bind to the same regions and induces similar changes on both intergenic RNAPII and target gene expression, we conclude it is very

likely the ER tag does not interfere with the analysis and FOXO3A3-ER faithfully mimics endogenous FOXO3.

5. It is not clear from the Materials and Methods section how many biological replicates were subjected to the ChIP-seq procedure. Have ChiP-seq data been obtained with chromatin from a single experiment with a single biological sample in each condition or from chromatin extracted from a number of biological replicates and pooled? This should be clarified.

The initial experiments were data from single experiments. However the 4h and 24h treatments in DL23 cells show very similar changes relative to untreated samples, therefore already providing two biological replicates of FOXO3 activation. In addition, the DL23 untreated sample shows very similar profiles to DLD1 treated and untreated profiles, providing de facto four biological repeats without FOXO3 activation. Data from RNAPII ChIP-seq, FOXO3 ChIP-seq and micro-array analysis were obtained separately, therefore the overlap between these experiments shows consistency in effects of FOXO3 activation.

In addition, we have now also performed RNAPII ChIP-seq experiments of DL23s, comparing activation of FOXO3A3-ER with 4OHT with effects of PKB inhibition. These experiments were performed in duplicates for each condition. Figure S10 shows the overlap between experiments, again showing consistency in effects of FOXO3 activation.

Regarding the FOXO3 ChIPs, initial data already showed a considerable overlap between intergenic RNAPII and FOXO3 peaks, which were performed separately (Fig 5B). Also only FOXO3 bound RNAPII peaks increase upon FOXO3 activation. In addition, in Figure S3 we can show consistent binding (n=3) to six regions specifically in DL23 cells upon 4OHT activation only, with two different antibodies. We validated FOXO3 binding to 15 more regions upon 4OHT activation and all 15 showed increased binding, making a total of 21/21 regions. As mentioned above, profiles from endogenous FOXO3 and the DBD fusion show increased signal in the same regions and therefore also provide verification of the profile.

Reviewer #2 (Remarks to the Author):

The title of the paper has to convey a message, but I am not sure that the current version has the balance quite right. Only about a third of the enhancers that the authors find to be regulated by foxo3a are distal to the genes that they regulate, although of course this finding is an interesting one.

The reviewer is correct in his/her conclusion with respect to our title and we have changed our title to cover more correctly the overall content of our paper. The title now reads "Genome wide analysis of FOXO3 target gene regulation through RNA pol II profiling".

It should be pointed out somewhere in the MS that the power of the induction system used comes with some downsides. Any cell-based study will have limitations in terms of the information gained about what happens in vivo in specific tissues, and the version of foxo3a used in the study was necessarily highly modified, with a tamoixfen-activated ligand-binding domain of the ER and a triple mutation to make it constitutively nuclear when activated. And who knows what other secondary modifications of the protein and co-factors are/are not present in this particular cell system with this particular version of the TF. As the authors point out in the Introduction to the paper, the mode of action of foxos is highly context-specific. These limitations probably mean that the data on the molecular mode of action of the TF are particularly robust and informative while the precise gene targets identified are less so. Of course it is reassuring to note that many of the targets identified are ones that would be expected from in vivo work and from the known functional outputs of the TF in vivo.

See also our answer to reviewer #1. We have now performed experiments showing binding of endogenous FOXO3 to similar regions upon PKB inhibition. PKB inhibition induced similar changes, on target gene expression, but also H3K27Ac and RNAPII induction at intergenic regions. Generally, the effects are similar but less upon PKB inhibition. Indeed, we need to discuss the downside of the system, but as shown in Figure 7A, the upside of the system is that we can also relate all changes we observe to FOXO3 activation specifically.

Introduction

There is an Oncogene review specifically on the role of foxos in ageing from Partridge and Bruening.

This has now been added

Results

The induction system needs a much more detailed description - I had to go to earlier publications to understand exactly what was being induced and how.

We have briefly summarized in the introduction the induction system we have used by recapitulating our previous work. Also in the light of using/describing in the revised manuscript the DNA binding region/NLS fused to ER as a control, the rationale for using and therefore the explanation of this system becomes even more evident.

Pol II occupancy was monitored only for annotated genes, in one sense this negates the power of sequencing, which is that it can detect unannotated transcripts. I assume the main reason this was done was that transcript changes were monitored with microarrays rather than with sequencing?

Indeed this was the case. This is a very valid suggestion and we will pursue RNA-sequencing for this reason. However, we consider this is outside of the scope of this study and will be published elsewhere.

Figure 1D It is not clear what the colour codes mean and the diagram needs more explanation in the figure legend.

The colour legend is now above the diagram. We have provided more information in the figure legend.

Figure 2B legend 'ration' should be 'ratio'. In Figure 2A the 4h and 24h induction data going towards the 3' end of the genes look progressively more different - is there some sort of genuine time course of binding here?

Indeed there seems to be a time course in which towards the end 24h inductions results in increased RNAPII signal. Possibly, 4 hours of induction is not long enough for RNAPII to progress towards the end of the gene. To test this hypothesis, we generated average profiles for 'short' and 'long' transcripts separately, but we could not find a difference in the profiles between these different categories. Alternatively, the increase in RNAPII occupancy towards the end of the gene represent molecules that fail to properly terminate and therefore a further increase upon longer stimulation can be observed. We can therefore not provide any statements regarding the mechanism responsible for the observed differences.

The sentence starting ' Interestingly, the subset of distal FOXO3 binding regions.....' refers to the data in Figure 3C which gives an average phastcons score. This needs explanation - exactly what has been measured here to make the deduction of 'conservation' of distal binding sites?

Phastcons scores provide base-by-base average conservation scores. We have included this in the figure legend.

Discussion

It is pointed out that pol II occupancy does not increase at motifs for TFs whose binding motifs are present in foxo3a bound regions. Could they be acting as co-repressors?

We could not identify any differences in RNAPII occupancy prior to FOXO activation and changes upon FOXO activation in regions categorized by presence of absence of specific

motifs. Motif presence therefore generally does not seem to enhance nor repress FOXO induced RNAPII induction, suggesting more complex regulation.

In the sentence starting 'GATA has been described to function.....' there is a 'for' missing before 'chromatin'

We thank the reviewer for noting this and we have inserted 'for'.

M and M

Heading should be: Quantification OF RNAPII occupancy

We thank the reviewer for noting this and we have inserted 'of'.

Recommendation

Accept with minor revision

Reviewer #3 (Remarks to the Author):

The work here presents a case for a relationship between FOXO3-mediated gene activation and RNA polymerase II (RNAPII) occupancy using ChIP-seq of both FOXO3 and RNAPII. The binding candidates showed enrichment for several known transcription factor sequence motifs. A substantial number of the candidate FOXO3 peaks were distal from their associated target gene and the authors demonstrate a concomitant increase in RNAPII occupancy, as well as H3K27ac enrichment (a widely reported marker of enhancer activity). Finally, the authors report 4C data suggesting a novel "spatial organization" between FOXO3 target genes and distal enhancers via chromatin looping. In short, the general interest is sufficient, the methodology is reasonable, but the results are not sufficiently convincing.

1)In general, peak-calling algorithms depend on arbitrary thresholds such as p-value or FDR cutoffs. Can authors go into more detail on how parameters were chosen to minimize false positives? Were input/mock controls used? Specifically, are the 9932 FOXO3 candidate-binding sites of high confidence, particularly in light of your observation that "not all regions fulfill the peak calling criteria"?

We have included details regarding parameters and controls in the materials and methods section.

2)How are the fig1A densities calculated? What are you units for the y-axes? Are these binned read counts, base pair counts?

Y-axis values represent tag coverage per base per 10^6 reads sequenced. We have included this in the figure legend.

3)In Figure 3A, is there any evidence of motif regulatory modules comprised of the enriched motifs? Since, FOX consensus motifs are present in only 45% of the peaks, do you have some intuition as to whether the remaining peaks are false positives or perhaps some alternate motif or sub-optimal FOX motif?

Lower stringency in mapping allowed identification of Forkhead motifs in an additional 16% of the peaks, a statement is included in the main text. Manual inspection suggested remaining peaks to also contain sub-optimal FOX motifs. Part of the peaks without Forkhead motifs can be additionally explained by indirect IP of a region looped to the primary binding site and to some extent by false positives, which cannot be completely excluded from any ChIP-seq experiment.

4)In Figure 4B, the distribution of the changes in RNAPII occupancy in some of the bins do not look normally distributed (long tail towards positive change). Perhaps a Kolmogorov-Smirnov test would be more appropriate?
5)For figures 5A and 5D, it also appears there are some non-normally distributed bins. Can a normality test be performed, or a K-S test in lieu of the unpaired t-test?

Indeed in both figure 4B and 5 distributions failed normality test and therefore we performed the non-parametric Mann-Whitney-Wilcoxon test for both figures.

6)In the 2nd to last sentence on page 16, is there a typo at "...shown to be transiently required chromatin unfolding...."

We thank the reviewer for noting this and we have inserted 'for'.

3rd Editorial Decision 30 October 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate your resubmitted study. As you will see, the referees felt that this work had substantially improved since its first version. The first reviewer, however, raises some important remaining concerns and make suggestions for modifications, which we would ask you to carefully address in a final revision of the present work. The editor notes that Reviewer #1's point #2 appears non-trivial, and seems to require some additional experimental work, supporting comparable expression levels of DLD-F3 and DLD1-DBD in cells.

In addition, when preparing your revised work please make sure all new large-scale datasets are including in the GEO deposit before resubmission.

Referee reports:

Reviewer #1 (Remarks to the Author):

The revised manuscript shows significant improvement and most issues have been addressed satisfactorily. I still have a few concerns though.

1. Average profiles of RNA Pol II enrichment at FOXO3 binding sites confirm that FOXO3 loading to these regions has a weak average impact. It could then be interesting to clusterize these regions according to Pol II signal in the presence of tamoxifen in order to identify regions where Pol II engagement is strongly enhanced by FOXO3 binding.

2. I appreciate the efforts put on resolving the issue of the ER moiety impact in the experimental system. However, data shown in Figures S7 and S8 raise questions. Indeed, it seems that ChIP-qPCR detects a much lower tamoxifen induction of fusion protein binding to selected FOXO3 binding sites in DLD1-DBD compared to DLD1-F3 cells (S7) and this is also observed for all DLD1-F3 aER peaks (S8). The authors should run a western blot using the anti-ER antibody to show that the two fusion proteins are expressed to comparable levels in DLD-F3 and DLD1-DBD cells. In addition, a heatmap and an average profile showing RNA Pol II signal -/+ OHT at the 2941 DLD1-DBD called peaks should also be included.

3. In the legend of Figure S8, the authors indicate that ´ Borders of identified peaks were increased to a minimum of 1 kb in size surrounding the peak centre [a] to determine overlapping peaks. What is the rationale for this ? What overlap numbers are obtained when bed files are left unchanged ?

Reviewer #2 (Remarks to the Author):

In this revised manuscript by Burgering and colleagues most of my previous comments were addressed.

1st Revision - authors' response 13 November 2012

We herewith send you our revised manuscript in which all of the remaining questions of reviewer # 1 are addressed. A detailed response is provided below. We have uploaded all additional datasets to GEO GSE35486 on the 5th of November 2012 and are awaiting final approval of the curators.

Taken together we wish to thank the reviewers for their comments that helped to further improve the quality of our manuscript and we hope that with these additional experiments and changes our manuscript is now suited for publication.

Reviewer #1 (Remarks to the Author):

The revised manuscript shows significant improvement and most issues have been addressed satisfactorily. I still have a few concerns though.

1. Average profiles of RNA Pol II enrichment at FOXO3 binding sites confirm that FOXO3 loading to these regions has a weak average impact. It could then be interesting to clusterize these regions according to Pol II signal in the presence of tamoxifen in order to identify regions where Pol II engagement is strongly enhanced by FOXO3 binding.

We have followed this suggestion, and we have created two classes of intergenic RNAPII peaks after FOXO3 induction based on changes in RNAPII occupancy status (up >0.6 fold or unchanged), and correlated this with changes in the adjacent gene (Figure S12A, the reverse analysis from Figure 7A). We hereby could show that an upregulated status of the intergenic FOXO3 bound genes is predictive for a higher induction of the adjacent gene, indeed suggesting that regions where RNAPII engagement is strongly enhanced by FOXO3 binding are more involved in target gene regulation.

2. I appreciate the efforts put on resolving the issue of the ER moiety impact in the experimental system. However, data shown in Figures S7 and S8 raise questions. Indeed, it seems that ChIP-qPCR detects a much lower tamoxifen induction of fusion protein binding to selected FOXO3 binding sites in DLD1-DBD compared to DLD1-F3 cells (S7) and this is also observed for all DLD1-F3 aER peaks (S8). The authors should run a western blot using the anti-ER antibody to show that the two fusion proteins are expressed to comparable levels in DLD-F3 and DLD1-DBD cells.

With respect to the above "The editor notes that Reviewer #1's point #2 appears non-trivial, and seems to require some additional experimental work, supporting comparable expression levels of DLD-F3 and DLD1-DBD in cells."

We have included a western blot (Figure S7A) of DLD1, DLD1-F3 and DLD1-DBD whole protein lysates, showing the expression levels of both fusion proteins with the ER antibody. This shows that the expression level of the DBD fusion is comparable with that of the full length FOXO3 fusion.

In addition, a heatmap and an average profile showing RNA Pol II signal -/+ OHT at the 2941 DLD1-DBD called peaks should also be included.

We have also included a heatmap and the average RNAPII signal at all intergenic DLD1-DBD peaks and, for completeness, also intergenic endogenous FOXO3 peaks (Figure S9C,D).

3. In the legend of Figure S8, the authors indicate that « Borders of identified peaks were increased to a minimum of 1 kb in size surrounding the peak centre » to determine overlapping peaks. What is

the rationale for this? What overlap numbers are obtained when bed files are left unchanged?

The exact location of the borders is less accurate for smaller peaks. We therefore increased the size to a minimum of 1 kb to prevent that binding to the same regulatory regions but with slightly different border coordinates are not identified as overlapping. We have repeated the analysis with unchanged bed files, which resulted in similar numbers (see below). Importantly, as shown in Figure S8B, the vast majority of non-overlapping peaks have increased ChIP signal in all 3 libraries, which was below stringent peak detection level. This suggests much higher actual overlap.

Analysis in manuscript:		**DLD1-F3 +4OHT**	**DLD1 +PKB inh.**	**DLD1-DBD +4OHT**	**total**
		aER	**aFOXO3**	**aER**	
DLD1-F3 +4OHT	**aER**	**9152**	**336**	**562**	**9932**
DLD1 +PKB inh.	**aFOXO3**	**319**	**454**	**275**	**935**
DLD1-DBD +4OHT	**aER**	**539**	**279**	**2238**	**2941**

18% of DBD peaks overlap with called FOXO3A3-ER peaks
34% of endogenous FOXO3 peaks overlap with called FOXO3A3-ER peaks

New analysis, with unchanged bed files:		**DLD1-F3 +4OHT**	**DLD1 +PKB inh.**	**DLD1-DBD +4OHT**	**total**
		aER	**aFOXO3**	**aER**	
DLD1-F3 +4OHT	**aER**	**9296**	**286**	**450**	**9932**
DLD1 +PKB inh.	**aFOXO3**	**286**	**480**	**270**	**935**
DLD1-DBD +4OHT	**aER**	**449**	**270**	**2322**	**2941**

15% of DBD peaks overlap with called FOXO3A3-ER peaks
31% of endogenous FOXO3 peaks overlap with called FOXO3A3-ER peaks