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Brk regulates wing disc growth in part via repression of Myc expression

Nikolaos Doumpas, Marina Ruiz-Romero, Enrique Blanco, Bruce Edgar, Montserrat Corominas and Aurelio A. Teleman

Corresponding author: Aurelio A. Teleman, German Cancer Research Center DKFZ

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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: Esther Schnapp

1st Editorial Decision 05 September 2012

Thank you for the submission of your manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, while the referees agree that the study is potentially interesting, they also all point out that it requires significant revision before it can be considered for publication here. The major concerns regard the missing statistical evaluation/validation of the ChIP-Seq data, the missing discussion of the data and a number of missing controls. Both referees 2 and 3 indicate that a negative control sample is required for the ChIP-Seq assay to validate the more than 1300 peaks. Both referees also remark that the novel Brinker antibody should be better characterized, given the high background staining. Referee 1 further mentions that in order to investigate Myc regulation by Brinker, myc transgenes should be generated (or may already exist according to referee 3) that are insensitive to Brinker regulation and these should be used in the rescue experiments. Along these lines, referee 3 suggests to use another growth regulator that is not a Brinker target as a negative control for the rescue experiment. Finally, both referees 1 and 2 point out that the claim that wing growth is restored in the rescue experiment is an overstatement, which needs to be tuned down.

From the referee comments it is clear that, as it stands, the technical quality of the study is

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low/unacceptable and publication of the manuscript in our journal can therefore not be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the length of the revised manuscript may not exceed 30,000 characters (including spaces, figure legends and references) and a maximum of 5 figures, plus 5 supplementary figures that must directly relate to their corresponding main figure may be presented. Shortening of the text may be made easier by moving parts of the materials and methods to the supplementary information. Please note that the materials and methods essential for the understanding of the experiments described in the main manuscript file must remain in the main manuscript file (for example the description of the ChIP-Seq assay). Please also include the sample size (n) in each figure legend.

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I look forward to seeing a revised version of your manuscript when it is ready.

REFEREE REPORTS:

Referee #1:

The manuscript submitted by Teleman and colleagues reports on a genome wide ChIP-Seq data of Brinker in imaginal wing discs of Drosophila. The authors then test the role of over-expression of two suspicious candidate genes, and conclude that these two genes play a major role in Brk dependent disc growth regulation. Although the work is interesting to a large readership, the authors should do some more work before the study can be published.

The authors also pick up many components of the Hippo pathway, and there are lots of interactions between Hippo and Dpp, but also Hippo and Myc. Is it possible that what the authors see is actually indirect regulation?

The authors conclude that much of the Dpp growth regulatory properties can be attributed to Myc and Bantam. Considering that there are 1300 other targets the authors describe in their work, it looks to me like this is an overstatement.

To really investigate the importance of the regulation of Myc by Brinker, the authors have to generate mutated myc rescue transgenes carrying altered Brk binding sites (and showing that these transgenes loose Brk regulation). This is especially important since Myc LEVELS do matter, as is well reported in the literature and also observed by the authors.

There is no discussion of any of the data. That should be changed. Didi the authors do any serious

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statistical evaluation of the ChIP-Seq data?

In Suppl Fig 3, not much of the endogenous expression pattern is seen. This data is not really nice, it could be done much better using fluorescent in situ labelings.

In Fig 3, it is not really clear which clones are shown. Their shape does not seem to fit which what is seen in the lower scale version.

Referee #2:

In this manuscript the authors show that myc is a target of the transcriptional repressor Brinker, which is the downstream effector of the Dpp signaling pathway. Further, they show that myc accounts in part for the growth regulation by brinker in the Drosophila wing imaginal disc.

While the immunohistochemistry analysis in the wing disc looks convincing the evaluation of the ChIPseq data seems very superficial (details see below). Thus, the ChIPseq data as it is presented here is of very questionable value and I would strongly urge the authors to either perform a much more sophisticated analysis on the data or dispense it altogether.

Minor Comments
1. In the introduction the authors should stay consistent with the use of gene and protein nomenclature.
2. It would be good to indicate in all figures the samples size (n) and how many biological or technical replicates were performed to collect the presented data.

Major Comments
1. General: ChIPseq data
The description of the ChIP experiment is very incomplete. How many discs were used as starting material, how many reads were recorded in the sequencing, what fraction of reads could be mapped (including parameters used for mapping) and what sequencing system was used (e.g. what company, single end or paired end)? Regarding the analysis it seems that the authors did not use any of the sophisticated available tools (e.g. MACS) to analyze their ChIPseq data. Instead the authors took a rather arbitrary cut off based on the smallest peak of a bona fide target of Brk. This analysis does not provide any statistical proof of the validity of their peaks. Either the authors should use their own analysis pipeline including sufficient statistical tools to proof the validity, or they should use well-established analysis pipelines (e.g. as demonstrated by the modENCODE consortium). Furthermore the authors do not provide any control sample (input DNA or mock-IP) as commonly used in the field.
Picking out myc from a total number of 1336 genes that contained peaks seems rather random given that only one peak barely made the authors arbitrary cut off.

2. Figure 1:
Panel A: It would be important to see the background peaks from a control sample to evaluate the validity of the peaks.
Panel C: It would be good to have a positive control region to compare the strength of induction caused by the different constructs to a bona fide target, since there is quite some significant variation in the observed induction. For example the background induction of peak 7 is higher than the overall induction of peak 3, 4 and 8.
Panel D: This panel could be moved into Supplementary Figure 2.

3. Figure 2:
Panel A&B: The authors should comment why region 2 and 3 (which was the single identified peak) do not cause a strong effect but region 4 (which is the most downstream) does. It seems that binding of Brinker does not correlate with transcriptional function very well.
Panel C: The observed effects are very different (and much weaker in C) to the corresponding

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effects shown in panel B although the binding fragment was trimerized. The authors should explain this observation in the text. Also the different scales in each chart make it hard for the reader to compare the data relative to each other.

4. Figure 3:
Panel B: It would be good to show a Brinker staining in TkvQD clones that illustrates changes in brk expression in these clones.
Panel D: The loss of Brinker leads to myc expressing clones. Previous work has shown that such clones become supercompetitors that overgrow the surrounding non-myc expressing tissue. Why does this effect not take place here? To this end it would be important to get detailed information about how clones were generated (e.g. timing and length of heat shock).
Panel E: Why are the two discs of different size? Are they of different age (which could account for the observed effect) or are the pictures taken at different magnification (if so, a scale bar should be included)?
Panel F: The in situ staining in F' does not look very convincing to me. There might be a slight effect visible but overall the disc appears overstained. The authors should try to get a better image that shows the effect more clearly. Why are the authors using PEPCK as a control? It would be more convincing to show a gene with stronger expression (e.g. CycB Fig. S3) and the proper controls.

5. Figure 4:
Row 2: Since myc is a growth promoting gene one would expect larger wings in the myc expressing strain. Why is this not the case? It would also be nice to show the phenotype of a nub>bantam wing.
Row 6: It is not entirely true that patterning is not restored since there are some veins visible in this wing that are completely absent in row 3-5. The text should be adjusted accordingly. The claim that a large part of growth is restored is an overstatement in my opinion, since the growth is restored from roughly 20% (row 3) to 45% (row 6). Thus, it only accounts for approximately 25% of overall growth while the wings do not even reach half of the wild-type size.

Supplementary Figure 1
Since the immunohistochemistry of the anti Brinker suggests a high background it would be important and informative to show a Western Blot using the Brinker antibody. This would give some further indication about the specificity and validity of the ChIPseq data.

Discussion
A proper discussion of the results is missing altogether. The single paragraph raises an interesting point in commenting on the cell competition connection of myc and dpp, however it is way too short and does not reflect on any of the data presented in the results section.

In summary, I think that the overall finding that myc is a target of brinker is novel and interesting to a broad readership. However, the presentation of the ChIPseq data as it stands does not live up to standards in the field. In addition, the claim that myc and bantam are the main targets that explain most of the growth effects seems to be an overinterpretation of the authors. Due to its experimental shortcomings the manuscript to me does not merit publication in EMBO journal in its current form but might be suitable after major revisions as pointed out above.

Referee #3:

The authors use ChIPseq to identify transcriptional targets of the Dpp pathway component Brinker. They identify dMyc as one gene that is bound and repressed by Brinker, and provide evidence that dMyc mediates some of the growth promoting effects of Dpp signaling. This finding connects two major growth controlling pathways, and as such makes a lot of sense (indeed, data suggesting such a link have even been published by one of the participating authors, as "data not shown" in Prober and Edgar 2002, Genes Dev. 12, 2286 - so the present manucript might also want to cite the earlier publication).

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Nevertheless, several of the experiments are not fully convincing on their own, and should be strengthened by the inclusion of additional controls. Specifically:
1. The whole project relies on a novel anti-Brinker antibody that is insufficiently characterized. As far as characterization goes, there is only one immunofluorescence picture of a wild type wing imaginal disc. While this picture shows a clearly graded anti-Brinker staining (which is consistent with the known Brinker mRNA distribution), it also displays high signal intensity in the central region of the disc (where Brinker protein levels should be very low) - suggesting that this antibody has significant background reactivity. At the very least, the authors need to show immunofluorescence pictures of wing discs containing (large) brinker mutant clones in the lateral region, and demonstrate that such clones retain no immunoreactivity.
2. The authors identify 1336 potential Brinker targets, but it is hard to assess the reliability of these ChIPseq data (unfortunately, my copy of the manuscript lacks Supplemental Tables 1 and 2, so I cannot comment on the specifics of these data). It would be important to establish how many of these peaks are bound by control antibodies - given that some of the co-authors have carried out several such ChIPseq experiments, they should have control data at their disposal; an ideal control would be a ChIPseq experiment of Brinker mutant wing discs (using an allele that survives to larval stages and produces large discs), but this might be asking too much.
Along the same lines: the authors later use relaxed criteria to find the Brinker binding sites #1 and #4 in the dMyc locus, and they use sequence analysis to find the binding site #2 - if they apply either of these criteria to the whole genome, how many Brinker targets are found? Clearly more than 1336 (which already corresponds to 10% of the genome), so the question arises how strong an argument one can make with these data for dMyc being a 'specific' Brinker target.
3. The expression pattern of dMyc is consistent with it being repressed by high levels of Brinker. However, this does not constitute a very strong argument for dMyc as a Brinker target, since other genes that were selected as examples for non Brinker targets show a similar distribution (e.g. melted, Fig. S3).
4. dMyc protein shows upregulation in Tkv*- or Brinker-mutant clones, which is consistent with the authors' claim. However, in several of the clones shown in Fig. 3 this upregulation is particularly strong at the border of the clone, raising an alternative possibility that it might be caused by the interface between Brinker-mutant and wild type cells (e.g. by a phenomenom linked to cell competition?), rather than by a cell-autonomous direct effect of Brinker on the dMyc gene.
5. The wing size defect caused by Brinker overexpression is partially rescued by co-expression of dMyc, whereas dMyc overexpression alone rather decreases wing size (presumably as a consequence of apoptosis, which is documented to follow from dMyc overexpression). This observation is also consistent with dMyc being downstream of Brinker, but doesn't prove it. The authors should also show to which extent a different growth regulator that is not targeted by Brinker (e.g. PI3K) rescues the Brinker overexpression phenotype - the prediction is that there would be at most additive effects, but no rescue.
Along the same line, another possible experiment would exploit published flies that carry a dMyc null mutation, but are kept alive by a ubiquitously expressed dMyc transgene; such flies reach similar sizes as wild type flies, but they are predicted to be less sensitive to overexpression of Brinker.

1st Revision - authors' response 15 November 2012

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Referee #1:

The manuscript submitted by Teleman and colleagues reports on a genome wide ChIP-Seq data of Brinker in imaginal wing discs of Drosophila. The authors then test the role of over-expression of two suspicious candidate genes, and conclude that these two genes play a major role in Brk dependent disc growth regulation. Although the work is interesting to a large readership, the authors should do some more work before the study can be published.

The authors also pick up many components of the Hippo pathway, and there are lots of interactions between Hippo and Dpp, but also Hippo and Myc. Is it possible that what the authors see is actually indirect regulation?

We do not think we are observing indirect regulation. We see binding of endogenous Brinker at the myc locus (Figure 2A), indicating a direct interaction. We also see that Brinker regulates expression of the four luciferase reporters derived from these myc enhancers (Figure 2B) - this experiment is done in S2 cells where Hippo signaling is not functional. Finally, these myc reporters lose regulation when we mutate the brk binding sites (Figure 2C) indicating once again that it is the direct binding of brinker to these genomic regions which imparts regulation. These data do not exclude the possibility which the reviewer points out that there may also be additional indirect regulatory mechanisms. Indeed, all of these pathways are inter-linked. Nonetheless, the Hippo components that are identified are all of the 'tumor suppressor' category, meaning that their suppression by Brinker would lead to increased growth and hence more Myc, not less Myc.

The authors conclude that much of the Dpp growth regulatory properties can be attributed to Myc and Bantam. Considering that there are 1300 other targets the authors describe in their work, it looks to me like this is an overstatement.

We have changed the wording to indicate that Myc is one of several mechanisms by which Brk regulates growth. Nonetheless, a significant fraction of the growth of brinker overexpressing discs is restored by providing solely Myc and Bantam (Figure 4).

To really investigate the importance of the regulation of Myc by Brinker, the authors have to generate mutated myc rescue transgenes carrying altered Brk binding sites (and showing that these transgenes loose Brk regulation). This is

especially important since Myc LEVELS do matter, as is well reported in the literature and also observed by the authors.

The reviewer is suggesting an experiment which is technically very difficult, as it would entail cloning the 50kb genomic region of myc and mutating the brk binding sites. These sites cluster into at least 3 regions, each one of which contains multiple brinker binding sites, meaning we would need to put a total of 11 point mutations into this large genomic region. Furthermore, and most importantly, this assumes we have identified ALL the brinker binding sites in these 50kb, which might not be the case (and we are not claiming). Indeed, the Brk ChIP-seq shows many smaller peaks throughout this region. Therefore, any additional brinker binding sites in the area would likely still impart regulation on the rescue construct.

The experiment suggested by the reviewer aims to show two things:
1. the brinker sites we have identified are functional
2. regulation of myc levels is one mechanisms by which brinker controls tissue growth.
The data we provide strongly support each of these two conclusions. The luciferase assays presented in Figures 2B and 2C show that these sites are functional and that mutating the brinker binding site abolishes regulation by Brinker. We provide a number of data showing that brinker regulates myc levels in vivo (Figure 3), and as the reviewer points out, it is well established that myc levels are important for growth. Finally, in Figure 4 we show that reconstituting myc expression rescues growth of a brinker overexpressing disc. Therefore although the experiment suggested by the reviewer would be elegant and would show these conclusions in an additional manner, we do not think it would give new insights compared to the data already provided.

There is no discussion of any of the data. That should be changed.

We have added more discussion. (Since the EMBO Reports short format combines Results and Discussion, discussion is provided in parallel as the data are presented.)

Didi the authors do any serious statistical evaluation of the ChIP-Seq data?

The ChIP-seq data were indeed statistically analyzed, and we have now added a description of this to the text. We

originally used two methods for identifying peaks – the Peakseq software from the Gerstein lab, as well as the more simple method of setting a flat threshold of 75 reads per region. Since the two methods give very similar results (presumably because the ChIP data are quite clean), we had decided in the original version of the manuscript to use the simple threshold method for data presentation. We have now replaced it with the Peakseq analysis, using a stringent p-score cutoff of 10^{-10}. The data are very similar, so that the biological conclusions are unchanged. We originally had 2249 peaks, we now have 2547. For instance, for validating peaks, we had randomly chosen a genomic region on the X chromosome (bp 5590283 to 5795911) which contained 8 peaks (Fig 1C). Seven of the eight are the same in the new analysis, whereas the 8th peak did not come through (although on average Peakseq identified more peaks than the flat threshold). Circa 80% of the original genes are in the new list, so that the gene ontology enrichment has not changed. Regarding myc, the Peakseq software identifies the same peak as the flat threshold does.

In Suppl Fig 3, not much of the endogenous expression pattern is seen. This data is not really nice, it could be done much better using fluorescent in situ labelings.

We have added to Supplemental Figure 3 in situs with fluorescent detection rather than histochemical detection. Although the results are the same using both methods (ptc>Brk suppresses myc expression but not expression of expanded or PEPCK), the fluorescent detection in our hands is less sensitive than histochemical detection. For this reason, despite our best intentions, we could not get decent fluorescent signals with the other probes (although we know that these genes are indeed expressed in the wing, and give signal with the histochemical method).

In Fig 3, it is not really clear which clones are shown. Their shape does not seem to fit which what is seen in the lower scale version.

We had included white boxes in Figures 3C", 3D" and 3E" indicating which clones are shown. We have made these boxes with thicker lines to make them more evident, and have added a note in the figure legend..

Referee #2:

In this manuscript the authors show that myc is a target of the transcriptional repressor Brinker, which is the downstream effector of the Dpp signaling pathway. Further, they show that myc accounts in part for the growth regulation by brinker in the Drosophila wing imaginal disc.

While the immunohistochemistry analysis in the wing disc looks convincing the evaluation of the ChIPseq data seems very superficial (details see below). Thus, the ChIPseq data as it is presented here is of very questionable value and I would strongly urge the authors to either perform a much more sophisticated analysis on the data or dispense it altogether.

We originally used two methods for calling peaks – a simple flat threshold, as well as a more sophisticated statistical analysis using the Peakseq software from the Gerstein lab. Since the two methods gave very similar results (presumably because the ChIP data are quite clean), we had decided in the original version of the manuscript to use the simple threshold method for data presentation. We agree with the reviewer that the statistical analysis is more appropriate, and have now replaced it with the Peakseq analysis, using a stringent p-score cutoff of 10^{-10}.

The results of the new analysis are very similar to the original ones, so that the biological conclusions are unchanged. We originally had 2249 peaks, we now have 2547. For instance, for validating peaks we had randomly chosen a genomic region on the X chromosome (bp 5590283 to 5795911) which contained 8 peaks (Fig 1C). Seven of the eight are the same in the new analysis, whereas the 8th peak did not come through (although on average Peakseq identified more peaks than the flat threshold). Circa 80% of the original genes are in the new list, so that the gene ontology enrichment has not changed. Regarding myc, the Peakseq software identifies the same peak as the flat threshold does.

Minor Comments

1. In the introduction the authors should stay consistent with the use of gene and protein nomenclature.

Fixed.

2. It would be good to indicate in all figures the samples size (n) and how many biological or technical replicates were performed to collect the presented data.

Done.

Major Comments

1. General: ChIPseq data

The description of the ChIP experiment is very incomplete. How many discs were used as starting material, how many reads were recorded in the sequencing, what fraction of reads could be mapped (including parameters used for mapping) and what sequencing system was used (e.g. what company, single end or paired end)? Regarding the analysis it seems that the authors did not use any of the sophisticated available tools (e.g. MACS) to analyze their ChIPseq data. Instead the authors took a rather arbitrary cut off based on the smallest peak of a bona fide target of Brk. This analysis does not provide any statistical proof of the validity of their peaks. Either the authors should use their own analysis pipeline including sufficient statistical tools to proof the validity, or they should use well-established analysis pipelines (e.g. as demonstrated by the modENCODE consortium). Furthermore the authors do not provide any control sample (input DNA or mock-IP) as commonly used in the field.
Picking out myc from a total number of 1336 genes that contained peaks seems rather random given that only one peak barely made the authors arbitrary cut off.

We have fixed the manuscript to address all these issues. In the Materials & Methods we have included a more detailed description of the ChIP-seq method, answering all the questions the reviewer asks (number of discs, how many reads, how many mapped, etc.). As mentioned above, we have also replaced our original analysis with a statistical call of peaks by the Peakseq software, comparing to input. Of the two possible controls for ChIP-Seq experiments (sonicated reverse-cross-linked chromatin or immunoprecipitation with a control IgG antibody) we normally use the first one, as we have found that the second approach generates little DNA and does not provide a good baseline for a peak calling control. This new analysis also identifies the same myc peak with a score $p<10^{-10}$.

2. Figure 1:

Panel A: It would be important to see the background peaks from a control sample to evaluate the validity of the peaks.

Done. This has been added as Supplemental Figure 2A, so as to not make the main figures too busy.

Panel C: It would be good to have a positive control region to compare the strength of induction caused by the different constructs to a bona fide target, since there is quite some significant variation in the observed induction. For example the background induction of peak 7 is higher than the overall induction of peak 3, 4 and 8.

We have added a positive control reporter alongside the reporters for Regions 1 and 7 as Supplemental Fig. 2B. As a positive control, we used a genomic region from Dad, which in our hands is the strongest Dpp target.

It is important to distinguish between two things:
1. the induction observed with the various reporters when Dpp signaling is activated or Brk is expressed, compared to the un-induced condition for each reporter, marked "-" in the figure. This induction is of relevance for our story, as it describes the response of the various reporters to Dpp signaling and to Brk.
2. the "background induction" which the reviewer is mentioning which one observes in the negative control conditions where Dpp signaling is not activated (marked "-" in the Figure) for the various reporters compared to the control reporter. Important to note is that S2 cells do not have endogenous Dpp signaling, which is why we activated Dpp signaling by expressing all the necessary components of the pathway – Tkv, mad and medea. The higher basal level of reporter #7 which the reviewer notes simply reflects the fact that this genomic region contains transactivating activity due to any number of binding sites for other transcription factors present in S2 cells compared to the control reporter. This, however, is not relevant to the present manuscript.

Panel D: This panel could be moved into Supplementary Figure 2.

Done. It is now Supplemental Figure 2D.

3. Figure 2:
Panel A&B: The authors should comment why region 2 and 3 (which was the

single identified peak) do not cause a strong effect but region 4 (which is the most downstream) does. It seems that binding of Brinker does not correlate with transcriptional function very well.

We have added a comment to the text. Here too, we have not normalized the luciferase results for each reporter individually (ie we did not set the uninduced expression for each construct to 1) since we think the current representation more fairly shows the expression of the various constructs relative to each other. Region 4 has a higher basal expression compared to the other regions. The induction, however, in response to Dpp sigaling is the same (9.1 - fold) as for Region 3 (9.8 – fold). The higher basal activity of Region 4 simply shows that other transcription factors bind to this region and activate expression, and has nothing to do with Brinker binding.

Panel C: The observed effects are very different (and much weaker in C) to the corresponding effects shown in panel B although the binding fragment was trimerized. The authors should explain this observation in the text. Also the different scales in each chart make it hard for the reader to compare the data relative to each other.

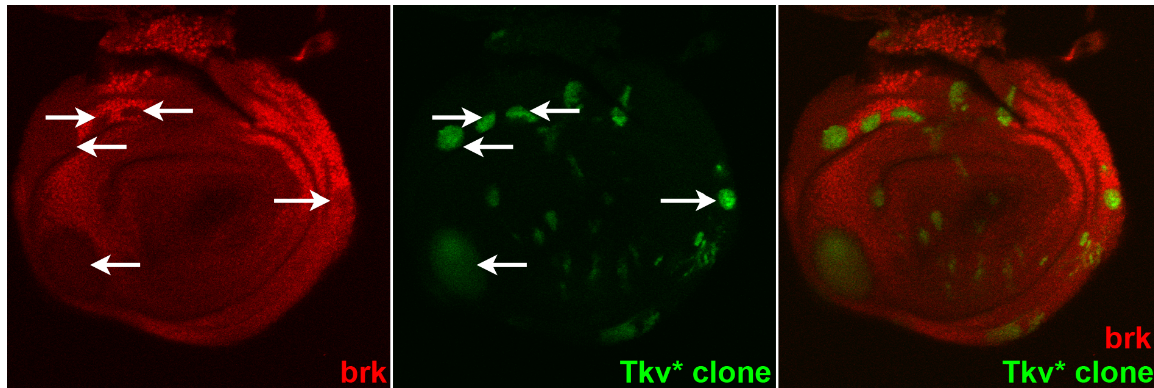
The complete Regions 2 and 4 (shown in Figure 2B) have 5 and 4 brinker binding sites each, respectively. For Figure 2C, we selected only a fragment of Regions 2 and 4, containing 2 brinker binding sites each (as shown in the figure, and described in the text). This was done in order to simplify the point mutagenesis. For this reason, the fragments were trimerized. We would like to note that once again, although the basal expression level in Fig 3C is lower than in Fig 3B, the induction is in the same order of magnitude (circa 9-fold). This makes sense since our trimerized reports in Fig 3C now have 3x2=6 binding sites each, while the reporters in Fig 3B have 5 or 4 binding sites. The low basal expression of the reporters in Fig 3C, which essentially contain only the Mad/Medea and Brk binding sites, reflects the fact mentioned above that S2 cells do not have endogenous Dpp signaling.

We have added a comment in the text to make this more clear.

4. Figure 3:
Panel B: It would be good to show a Brinker staining in TkvQD clones that

illustrates changes in brk expression in these clones.

This has already been published, but we add it here for the reviewer:



As expected, TkvQD clones display reduced Brinker expression. Since this is already published, we think it would be a bit awkward to publish it again, but we could add it as a supplemental figure if the reviewer thinks it is necessary.

Panel D: The loss of Brinker leads to myc expressing clones. Previous work has shown that such clones become supercompetitors that overgrow the surrounding non-myc expressing tissue. Why does this effect not take place here?

Brinker loss-of-function clones do indeed grow fast and large – this has also been published (PubMed ID 10052457). This is also evident in Figure 3D': lateral Brk loss-of-function clones (which show a large difference in Brk levels compared to neighboring tissue) are larger than the medial clones (where Brk is not highly expressed anyways).

To this end it would be important to get detailed information about how clones were generated (e.g. timing and length of heat shock).

We have added this information into the Supplemental Materials & Methods.

Panel E: Why are the two discs of different size? Are they of different age (which could account for the observed effect) or are the pictures taken at different magnification (if so, a scale bar should be included)?

The discs are of slightly different age.

Panel F: The in situ staining in F' does not look very convincing to me. There might be a slight effect visible but overall the disc appears overstained. The authors should try to get a better image that shows the effect more clearly. Why are the authors using PEPCK as a control? It would be more convincing to show a gene with stronger expression (e.g. CycB Fig. S3) and the proper controls.

We have added fluorescent in situs as Supplemental Figure 3B-C which show the same result as panels F, but with less background.

In our hands, PEPCK seems to be strongly expressed in the wing disc, since the signal develops robustly. We have moved the PEPCK negative control to Supplemental Figure 3, together with all the other negative control probes, giving a consistent picture of no effect regardless of strength of expression.

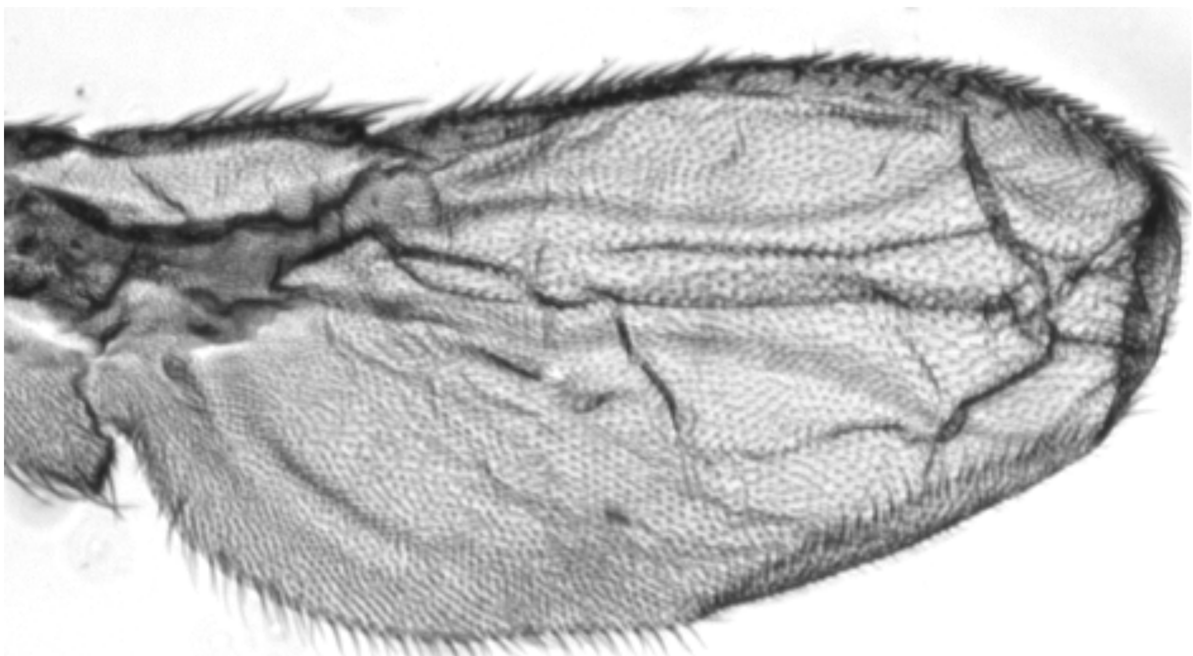
5. Figure 4:
Row 2: Since myc is a growth promoting gene one would expect larger wings in the myc expressing strain. Why is this not the case? It would also be nice to show the phenotype of a nub>bantam wing.

We do not know why overexpressing myc in the wildtype background does not make wings larger, however we have repeated one more time this experiment and reproduced the result, so the result is true (Supplemental Figure 4). (Reviewer #3 suggests this might be due to induction of apoptosis, which has been documented to follow Myc overexpression.)

We have included nub>bantam wings in Supplemental Figure 4. The level of bantam overexpression used in our experiments causes mild overgrowth.

Row 6: It is not entirely true that patterning is not restored since there are some veins visible in this wing that are completely absent in row 3-5. The text should be adjusted accordingly.

These wings actually do not have veins. What is seen in Row 6 are actually folds of the tissue. This can be seen on a higher magnification picture:



We have now added higher magnification images of two rescued wings as Figure 4B to make this point more clearly.

The claim that a large part of growth is restored is an overstatement in my opinion, since the growth is restored from roughly 20% (row 3) to 45% (row 6). Thus, it only accounts for approximately 25% of overall growth while the wings do not even reach half of the wild-type size.

We have replaced the word "large" with "significant". Considering that there seem to be >1500 target genes for Brinker, we think it is quite remarkable that only 2 of them combined cause quite a strong rescue.

Supplementary Figure 1
Since the immunohistochemistry of the anti Brinker suggests a high background it would be important and informative to show a Western Blot using the Brinker antibody. This would give some further indication about the specificity and validity of the ChIPseq data.

The ability of an antibody to recognize a protein on a Western blot, where the proteins are denatured, does not correlate very well with its ability to recognize a protein in situ or by IP. (Indeed, this is usually a pain because an antibody may work great on a western, but not for IPs and tissue stainings.) Instead, to address this point, we now provide an antibody staining of a brinker mutant wing disc (Supplemental Figure 1B') which shows that the

antibody is quite clean.

Discussion

A proper discussion of the results is missing altogether. The single paragraph raises an interesting point in commenting on the cell competition connection of myc and dpp, however it is way too short and does not reflect on any of the data presented in the results section.

We have extended the discussion.

In summary, I think that the overall finding that myc is a target of brinker is novel and interesting to a broad readership. However, the presentation of the ChIPseq data as it stands does not live up to standards in the field. In addition, the claim that myc and bantam are the main targets that explain most of the growth effects seems to be an overinterpretation of the authors. Due to its experimental shortcomings the manuscript to me does not merit publication in EMBO journal in its current form but might be suitable after major revisions as pointed out above.

Referee #3:

The authors use ChIPseq to identify transcriptional targets of the Dpp pathway component Brinker. They identify dMyc as one gene that is bound and repressed by Brinker, and provide evidence that dMyc mediates some of the growth promoting effects of Dpp signaling. This finding connects two major growth controlling pathways, and as such makes a lot of sense (indeed, data suggesting such a link have even been published by one of the participating authors, as "data not shown" in Prober and Edgar 2002, Genes Dev. 12, 2286 - so the present manucript might also want to cite the earlier publication).

We have added a citation to this earlier report.

Nevertheless, several of the experiments are not fully convincing on their own, and should be strengthened by the inclusion of additional controls. Specifically:

1. The whole project relies on a novel anti-Brinker antibody that is insufficiently characterized. As far as characterization goes, there is only one immunofluorescence picture of a wild type wing imaginal disc. While this picture shows a clearly graded anti-Brinker staining (which is consistent with the known Brinker mRNA distribution), it also displays high signal intensity in the central region of the disc (where Brinker protein levels should be very low) - suggesting

that this antibody has significant background reactivity. At the very least, the authors need to show immunofluorescence pictures of wing discs containing (large) brinker mutant clones in the lateral region, and demonstrate that such clones retain no immunoreactivity.

We have added data in Supplemental Figure 1 that show that this antibody is actually very clean and does not have significant background reactivity (detailed below). We agree with the reviewer that previously published expression data (either in situs or lacZ reporters) show a more steep gradient from lateral to medial regions of the wing disc than what we see with our antibody. However, this is the first time that, to our knowledge, Brinker protein has been detected, and it is well known that protein levels do not correlate linearly with mRNA levels due to both translational and post-translational mechanisms (ie the protein gradient need not be as steep).

We provide in Supplemental Figure 1B and B' images of wildtype and Brinker[XA] discs (which overgrow), stained with our anti-Brinker antibody. Both the stainings and the confocal imaging were done in parallel and with identical parameters for the two genotypes. Clearly, almost all the signal is gone in the Brinker loss-of-function discs, indicating the antibody does not have significant background reactivity.

Additionally, to specifically address the reviewer's comment regarding Brinker protein in medial regions of the disc, we knocked-down Brinker expression in the dorsal region of the disc using apterous-GAL4>uas-brinker[RNAi] (Vienna KK line). Please note that this is not a complete brinker loss-of-function, but rather a hypomorphic condition. In Supplemental Figure 1C one can clearly see that also the more medial staining is strongly blunted in the dorsal pouch, indicating that the more medial signal is also specific (in agreement with Suppl. Fig. 1B-B').

2. The authors identify 1336 potential Brinker targets, but it is hard to assess the reliability of these ChIPseq data (unfortunately, my copy of the manuscript lacks Supplemental Tables 1 and 2, so I cannot comment on the specifics of these data). It would be important to establish how many of these peaks are bound by control antibodies - given that some of the co-authors have carried out several such ChIPseq experiments, they should have control data at their disposal; an

ideal control would be a ChIPseq experiment of Brinker mutant wing discs (using an allele that survives to larval stages and produces large discs), but this might be asking too much.

We have now added data to address this issue. There are two possible controls for the ChIP-seq: sonicated reverse-cross-linked chromatin ("input") or immunoprecipitation with a control IgG antibody. We normally use the first one, as we have found that the second approach generates little DNA and does not provide a good baseline for a peak calling control. For this reason, we now provide an analysis of the peaks relative to normalized input using the Peakseq statistical software, and use a significance cut-off of $p<10^{-10}$. As an independent validation of the peaks, we tested randomly selected peaks for brinker responsiveness by luciferase assay, and all the peaks tested are responsive (Figure 1C).

Along the same lines: the authors later use relaxed criteria to find the Brinker binding sites #1 and #4 in the dMyc locus, and they use sequence analysis to find the binding site #2 - if they apply either of these criteria to the whole genome, how many Brinker targets are found? Clearly more than 1336 (which already corresponds to 10% of the genome), so the question arises how strong an argument one can make with these data for dMyc being a 'specific' Brinker target.

Indeed, the finding that Brinker binds many sites throughout the genome is an unexpected, useful and novel finding that results from our work. That said, many transcription factors have similar numbers of binding sites genome-wide. For instance, our previous work on FOXO identified >630 sites genome-wide. This should not call into question the validity of the binding sites, or the fact that the target genes are indeed target genes.

3. The expression pattern of dMyc is consistent with it being repressed by high levels of Brinker. However, this does not constitute a very strong argument for dMyc as a Brinker target, since other genes that were selected as examples for non Brinker targets show a similar distribution (e.g. melted, Fig. S3).

We agree. The reciprocal expression pattern of myc and Brinker is only the starting point, raising plausibility of this inhibitory interaction. The data proving that Myc is repressed by Brinker are presented in the rest of Figure 3:
1. Brinker loss-of-function clones show increased Myc protein levels, indicating endogenous Brinker is repressing

Myc (Figure 3D)
2. Thickveins gain-of-function clones, which have reduced Brinker levels, have increased Myc protein and Myc mRNA levels (Figure 3B and 3C)
3. Transiently expressing Brinker medially in the wing disc reduces Myc protein and mRNA levels (Figure 3E, F and G).

4. dMyc protein shows upregulation in Tkv*- or Brinker-mutant clones, which is consistent with the authors' claim. However, in several of the clones shown in Fig. 3 this upregulation is particularly strong at the border of the clone, raising an alternative possibility that it might be caused by the interface between Brinker-mutant and wild type cells (e.g. by a phenomenom linked to cell competition?), rather than by a cell-autonomous direct effect of Brinker on the dMyc gene.

We have several lines of evidence suggesting that the regulation of Myc by Brinker is direct:
1. We see binding of endogenous Brinker to the Myc genomic locus in wing discs by ChIP.
2. Luciferase reporter assays in S2 cells show that these genomic loci are indeed transcriptionally repressed by Brinker.

As the reviewer notices, there is an additional modulation of myc expression that causes particularly high levels at the border of some clones. Recently, discussing these data with Laura Johnston, she mentioned she sees similar effects on Myc when making loss-of-function clones for other Myc regulators, suggesting this is something general for Myc (having to do with transcriptional feedback loops on myc), rather than Brinker. We have added some text to the discussion in this regard.

5. The wing size defect caused by Brinker overexpression is partially rescued by co-expression of dMyc, whereas dMyc overexpression alone rather decreases wing size (presumably as a consequence of apoptosis, which is documented to follow from dMyc overexpression). This observation is also consistent with dMyc being downstream of Brinker, but doesn't prove it. The authors should also show to which extent a different growth regulator that is not targeted by Brinker (e.g. PI3K) rescues the Brinker overexpression phenotype - the prediction is that there would be at most additive effects, but no rescue.

We now provide additional data as Supplemental Figure 4 showing that the reviewer's prediction is exactly correct. Expression of PI3K increases the size of wildtype wings (by circa 20% in our conditions) but does not rescue the size of Brinker expressing wings (if anything, it makes them

slightly smaller).

Along the same line, another possible experiment would exploit published flies that carry a dMyc null mutation, but are kept alive by a ubiquitously expressed dMyc transgene; such flies reach similar sizes as wild type flies, but they are predicted to be less sensitive to overexpression of Brinker.

This experiment proposed by the reviewer is essentially the same as the one we present in Figure 4, except that in our case we maintain Myc expression using a UAS-construct whereas in the experiment proposed by the reviewer Myc expression is maintained via a ubiquitous promoter. We do not see how this experiment would prove that "Myc is downstream of Brinker" more than Figure 4 does. The proof that Myc is downstream of Brinker comes from the ChIP-seq data, the luciferase assays with wildtype and brinker-site-mutated reporters and the clonal analyses presented in Figure 3 showing that myc levels and expression are regulated by Brinker.

2nd Editorial Decision 10 December 2012

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees. As you will see, all referees support publication of the study in EMBO reports now. However, referees 2 and 3 still have a few suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

I think all comments by referee 2 should be addressed. Given that the manuscript has a total of 4 figures, the single figures can be slightly bigger. If you feel that it is feasible, it would also clearly strengthen the study if you performed the experiment suggested by referee 3, and provided an independent confirmation that Brk binds to the Myc locus.

In addition, as I told you in my last letter, the reference style of the manuscript needs to be changed into a numbered one. I also noticed that the error bars are not specified in SF4 and that Figures 3, SF1, SF3 are missing scale bars. Please include these missing information.

I look forward to seeing a new revised version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #1:

The authors have carefully addressed the points that were raised and have answered them adequately.

Referee #2:

In this manuscript Doumpas et al. show that myc is a direct target of the transcriptional repressor Brinker, which is a downstream effector of the Dpp signaling pathway. Further, they show that myc accounts in part for the growth regulation by brinker in the Drosophila wing imaginal disc.

The revised manuscript improved significantly compared to the initial version in several points:

- characterization of the novel Brinker antibody
- statistical validation of the ChIPseq data
- improved explanations in the text and the figure legends
- improved discussion

I still have some minor comments that should be addressed before final acceptance. These won't require additional experiments but rather rearrangement of figures and text:

- The authors should briefly mention the applied statistical parameters in the corresponding results section (MINFDR and PVALTRESH).
- Fig.1A I would like to see the background traces in the figure rather than the supplementary figure 2A to give the reader a fair representation of the data.
- Fig.1C I would like to see the positive control from the Dad locus in the figure itself rather than the supplementary figure 2B to give the reader a fair representation of the data.
- In contrast to the authors I think that a normalization of the individual luciferase induction data would be a better representation (or shown as fold change for each construct) since it makes it easier to compare the effect of "+Dpp" and "+Brk" across the board. As the authors pointed out, the background induction level (probably caused by unrelated factors that bind to the same region) is not of particular interest to the presented manuscript.

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- Figure 3: the panel that shows Brinker levels in Tkv clones (included in the response letter) would serve well as a supplementary figure for a reader that is not so well informed about dpp-signaling.
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- Fig. 4: I would definitively want to have the nub>ban wing image and data added here rather than in Fig. S4.

Overall, I feel that the presented data that establishes myc as a brinker target gene together with the genome wide ChIPseq data, and the novel Brinker antibody will be of great interest to a broad readership. Thus, I believe that the manuscript can be accepted for publication with the indicated modifications.

Referee #3:

This manuscript has two principal parts: the first consists of the genome-wide identification of Brk target genes, the second of the characterization of Myc as a Brk target. The first part is not very convincing, but the second part is believable and should be sufficient to allow publication.
About the first part - the ChIPseq experiment: As is now shown in Sup. Fig. 1, the new affinity-purified antibody against Brk seems to be specific and well suited for IF (and hopefully ChIP). Also, the luciferase reporters assays show that several of the newly identified Brk binding sequences can respond to Dpp & Brk, at least when they're plasmid-bourne, and in S2 cells. Nevertheless, there are several aspects of the ChIP data that do not convince me. First, the authors write in the Methods section that less than 5 million out of 36 million reads could be mapped to the genome; such an extremely low fraction of mappable reads indicates a low quality of the sample - and hence calls into question any statistical analysis of the remaining mappable reads (unless there is a different explanation for this low fraction of good reads, e.g. an overabundance of contaminating DNA in the library, for example from salm sperm). Second, the input sample shown in Sup. Fig. 2A shows massive noise and big peaks in the input sample (some of which are as high as the "positive" peaks in the Brk-ChIP). A certain degree of noise is common in ChIPseq samples, but usually most of this background noise is reflected in the experimental sample (which then also contains the "real" peaks in addition). Here however, the input and the ChIPseq lanes look quite different. Given this difference, the PeakSeq software is likely to find peaks in the Brk ChIPseq sample that are "statistically significant" as compared to the input sample. To find out how meaningful the number of thus identified peaks is, the authors need to run PeakSeq "in reverse", i.e. determine how many such "statistically significant" peaks are found in the input sample when compared to the ChIPseq sample. If this latter number even only approaches 2547, I would not trust the list of identified Brk binding sites. Third, in my initial review I have asked how many Brk target genes the authors would identify if they applied the relaxed criteria genome-wide that they use for the Myc locus, i.e. relaxed the PeakSeq threshold to $p<0.01$, and also included regions that only fulfilled certain sequence criteria (without necessarily showing a peak in the Brk ChIPseq). They authors have not answered this question. Instead they have argued that, if the number of Brk binding sites is large, this does not mean that it has to be wrong, and further that other transcription factors such as FOXO bind to >630 sites. To this I can say that the number of claimed Brk binding sites defined under stringent criteria (2547) is already 4 times the number of FOXO sites, and if the criteria for binding site identification are relaxed, this number will increase further - which would put Brk into an altogether different category than FOXO or any other typical transcription factor. I agree with the authors that Brk could theoretically bind to an even higher number of sites - but this would be quite unusual for a transcription factor (unless it belongs to the basal transcription machinery) and the authors would need to invest more effort to show that Brk indeed has such an unusual behaviour; e.g. demonstrate that cells contain enough Brk protein to bind to all of these sites, and show that alterations of Brk levels affect the expression of so many genes.
For these reasons, I am concerned that many (if not most) genes on the list of Brk targets may be false positives - and hence that the list is of limited value, although it obviously does contain bona fide Brk targets (such as Dad and Salm).
As to the second part - the characterization of Myc as a Brk target. In light of my doubts about the original ChIPseq data, I encourage the authors to confirm the binding of Brk to the Myc locus in an

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independent experiment, e.g. by carrying out ChIPs from control and Brk-mutant flies and comparing the specific signals for the Myc locus. For the rest of this part I have nothing to criticize.

2nd Revision - authors' response 14 December 2012

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The authors have carefully addressed the points that were raised and have answered them adequately.

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Done.

- Fig.3: I would add the fluorescence in situ image (Fig. S3 B/C) to panel E&F, it would not require additional space if the panels are arranged pairwise vertically and would add additional independent support of the authors claim.

We would not like to separate Fig S3B/C myc in situs from Fig S3C expanded and PEPCK in situs (the negative controls), and all the panels together do not fit in the main figure. Since Fig S3 B/C simply shows the same data as the main Figure 3F, F', except using a different readout technique, we think it is reasonable for the readership to look at the supplementary figure if they are interested.

- Fig. 4: I would definitively want to have the nub>ban wing image and data added here rather than in Fig. S4.

Done.

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The fraction of mappable reads is similar to what we get when performing ChIP with other antibodies using wing discs as starting material (Perez-Lluch et al. 2011). Moreover, the modENCODE consortium has reported similar numbers for other TFs (e.g. Sin3A - modENCODE_3232, between 6868968 and

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7138052 mappable reads or Disco - modENCODE_3235, between 2712815 and 10915091 reads) in three replicates.

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There seems to be a misunderstanding of the "input control". This is not a no-antibody control. This is sequencing performed on the sheared chromatin, which is the input for the ChIP, and it is performed to control for uneven shearing and read depth throughout the genome. Since we use the same amount of DNA for sequencing the ChIP as the input control, on average the peaks will be the same height between the two samples. This is in contrast to a ChIP with no antibody, which in our hands gives no detectable DNA, and hence nothing to sequence. If we were to do the sequencing on this empty tube, we would also get no/low peaks.

Here however, the input and the ChIPseq lanes look quite different. Given this difference, the PeakSeq software is likely to find peaks in the Brk ChIPseq sample that are "statistically significant" as compared to the input sample. To find out how meaningful the number of thus identified peaks is, the authors need to run PeakSeq "in reverse", i.e. determine how many such "statistically significant" peaks are found in the input sample when compared to the ChIPseq sample. If this latter number even only approaches 2547, I would not trust the list of identified Brk binding sites. Third, in my initial review I have asked how many Brk target genes the authors would identify if they applied the relaxed criteria genome-wide that they use for the Myc locus, i.e. relaxed the PeakSeq threshold to $p<0.01$, and also included regions that only fulfilled certain sequence criteria (without necessarily showing a peak in the Brk ChIPseq). They authors have not answered this question. Instead they have argued that, if the number of Brk binding sites is large, this does not mean that it has to be wrong, and further that other transcription factors such as FOXO bind to >630 sites. To this I can say that the number of claimed Brk binding sites defined under stringent criteria (2547) is already 4 times the number of FOXO sites, and if the criteria for binding site identification are relaxed, this number will increase further - which would put Brk into an altogether different category than FOXO or any other typical transcription factor. I agree with the authors that Brk could theoretically bind to an even higher number of sites - but this would be quite unusual for a transcription factor (unless it belongs to the basal transcription machinery) and the authors would need to invest more effort to show that Brk indeed has such an unusual behaviour; e.g. demonstrate that cells contain enough Brk protein to bind to all of these sites, and show that alterations of Brk levels affect the expression of so many genes.
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This number of Brk target genes (2547 binding sites that

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correspond to 1671 genes) is not unusual for a transcription factor. In fact, the modENCODE web site reports similar (or even higher) numbers for several TFs: disco (1672 genes, modENCODE_3235), cabut (1621 genes, modENCODE_3825) or exd (7074 genes, modENCODE_3824).

3rd Editorial Decision 17 December 2012

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

Please, send me a brief 'Author Contributions' statement, which I will add to your article file.

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