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RNA polymerase II kinetics in *polo* polyadenylation signal selection

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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.)

1st Editorial Decision 26 March 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. I have now had an opportunity to read your manuscript carefully and I have also discussed it with an external Editorial Advisor with suitable expertise as well as my editorial colleagues and I am sorry to say that we cannot offer to publish the current study in The EMBO Journal.

I appreciate that you have described the role of alternative polyadenylation of Polo in regulating abdomen development. You generate flies with one of the two-polyadenylation sites missing, these flies mainly produce either the long or short Polo transcripts. Both transcripts can rescue the larval lethal phenotype in a strong hypomorphic polo background, however, only in the absence of the longer isoform do the flies die at the pupal stage, with escaper flies displaying a defective abdominal morphology. This seems to be due to decreased proliferation of histoblasts during the pupal stage in the absence of the longer isoform. This results in decreased Polo protein levels, which does not seem to be due to altered mRNA stability, but perhaps due to decreased translational efficiency of a long isoform reporter. In addition there is also evidence that the Polo protein can autoregulate polyadenylation site selection, since over-expression of Polo favours the shorter isoform and thereby decreasing protein levels.

However, as you discuss in the manuscript, it was previously known that Polo has two different

variants caused by alternative polyadenylation, and in other contexts alternative polyadenylation has been shown to occur during development. In addition, Suppressor of forked has been shown to autoregulate its 3'UTR processing and alternative polyadenylation has been shown to affect the translational efficiency of transcripts. Therefore, while I appreciate that you have nicely demonstrated an example of the role of alternative polyadenylation during development, after discussing this study with an external advisor we find that overall it does not provide sufficient new insight into the role of polyadenylation for the manuscript to be further considered for the EMBO Journal.

Please note that we publish only a small percentage of the many manuscripts that we receive at the EMBO Journal, and that the editors have been instructed to only subject those manuscripts to external review which are likely to receive enthusiastic responses from our reviewers and readers. As in our carefully considered opinion, this is not the case for the present submission, I am afraid our conclusion regarding its publication here cannot be a positive one. I am sorry to have to disappoint you on this occasion.

Yours sincerely,

Editor
The EMBO Journal

Resubmission 21 July 2010

May I urge you to reconsider this decision for this now revised and extended paper.

I consider this new paper to be important for the following reasons.
It is abundantly clear that alternative polyadenylation (APA) is an key aspect of gene regulation in mammals. However the mechanistic and biological functions of this process are still only poorly understood. This study on Drosophila polo gene APA site now provides both mechanistic and biological data that greatly extends published work on APA.

1) First it is beautifully shown here that only one of the two alternative polo pA signals (pA2) can be used to allow proper fly development. This is based on a full developmental analysis of transgenic flies with only one of either pA signal.

2) The mechanism of pA site choice is shown here to depend on Pol II transcription kinetics. If Pol II transcription is slowed down then the 1st pA site site is preferentially used. The data goes on to show that mRNA using the 1st pA signal is inefficiently translated.

3) An intricate autoregulatiory process is uncovered here. Thus Polo overexpression switches APA to the 1st pA signal so generating selectively only poorly translated pA1 polo mRNA.
In short it is clear to me that polo provides a paradigm for APA regulation and function. Surely this is exactly the type of paper that your journal needs to publish!

2nd Editorial Decision 24 September 2010

Thank you for submitting your manuscript for consideration at The EMBO Journal. I apologise for the unusual length of time that it has taken to have your study evaluated but I have now received the final report from the three referees that have evaluated your study. Overall the referees provide mixed recommendations with referees #1 and #3 being more positive regarding publication than referee #2. While referee #3 clearly appreciates the demonstration of the biological context of the alternative polyadenylation choice, both referee #1 and #2 raise concerns in the lack of link between the elongation rate of the polymerase, alternative poly(A) site selection and a molecular phenotype,

which should be extended. It is also important to generalize the concept of polymerase elongation rate and alternative poly(A) site choice. Should you be able to address these issues we would be happy to consider a revised version of the manuscript.

I should remind you that it is EMBO Journal 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. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This paper addresses two distinct questions: 1. Does the rate of transcription elongation affect the choice of polyadenylation site? and 2. is there a biological effect of polyA site choice in the polo gene? The data generally support that the answer to each question is yes. However it is not clear if there was an attempt to link these two observations; is there a biological effect of the change in polo expression in the mutant containing the "slow" polymerase mutant?

The data on the slow polymerase is restricted to a single transcript. To make definitive conclusions that the speed of the polymerase affects polyA site choice some additional alternative polyA sites need to be tested. To make the general conclusions the authors want to make requires at least 5 example of alternatively polydenylated transcripts. transcripts.

Specific comments:

1. The organization of the paper at the start is confusing, since they start by discussing Fig. 2. It would be better to start the paper with Fig. 2, and the diagram of the gene and the two polyA sites. The Northern blot shows they are used about equally under normal conditions. It would be useful to have a Northern also for the C4 mutant, although the PCR in Fig. 2C is convincing.

2. The interpretation of the CHIP data is not clear. In the wild-type there is a dramatic drop after the first polyA site, although the data shows that equal numbers of long and short transcripts are made. It is not at all clear how one can explain that drop. The authors should comment on the size of the DNA fragments obtained after shearing for the chip experiments, and how it compares to the size and separation of the different probes. A scale on Fig. 1A and/or 2A would be helpful. For example, the distance between probes 6 and 7 is likely less than 200 nts, which probably overlaps with the size of the DNA fragments obtained in the CHIP protocol. The "slow" polII gives more polydenylation at site 1, which could lead to subsequent termination before site 2. Thus the CHIP data is the opposite of what one would intuitively expect and the authors need to discuss this.

3. How the two polyA site usage is "quantified" by qPCR is not clear. Any primer used to amplify the short transcript will also amplify the long transcript. If it is done by 3' RACE, then it is certainly qualitative and not quantitative.

4. near end page 8, When they say that the change in polyA site selection has no effect on expression, do they mean mRNA or protein?. From the rest of the paper, the protein levels should be

affected if the short polyA form is the major one, and it doesn't get translated well. The western blot in supp. Fig. 1B shows no effect on the protein. Since this is unexpected given the change in polyA site usage this data should be included in Fig. 1, rather than in supplemental data.

5. The "cryptic" polyA site in Fig. 2C, needs to be documented or removed from the paper. If it really is a small fraction of the transcripts, how do the authors know it isn't present in the wild-type also?

6. The Western blot shown in Fig. 5C does not support the graph in Fig. 5C. There is reduction in both tubulin and GFP-polo on overexpression of polo and certainly the data don't look like the 5-fold change reported in the graph.

Referee #2 (Remarks to the Author):

The authors have examined alternative polyadenylation in the Drosophila gene encoding the polo kinase, an important cell cycle regulator.

They find that a mutant RNA polymerase II with a lower elongation rate leads to an increase in the use of the first of two alternative polyadenylation sites. This supports a first come, first served model. They also show that use of the second site is essential for fly development. RNAs terminating at the second site are translated more efficiently.

The data of this paper are technically mostly convincing. One major flaw is mentioned below.

There is little connection between the part of the paper concerning the pol II mutant and the rest, as the pol II mutant flies do not seem to have a phenotype that could be explained by a decreased use of the second poly(A) site. There is also not too much mechanistic insight in the paper. Why is the longer RNA translated more efficiently?

Specific comments:

1. p. 8: I do not see how a reduced elongation rate should reduce the number of pol II molecules on the gene. If anything, an increase would be expected. A decrease could be explained by (i) a lower initiation frequency (ii) an increase in premature termination and (iii) a reduced recovery during the ChIP procedure. I do not think that the paper depends on an explanation, but none is preferable to one that does not make sense.

2. p. 9: Could the authors give us a rough idea what 'a small proportion of transcripts ending at a cryptic pA site' means? How small?

3. p. 10 and Fig. 2B: The expression of the polo transgenes is shown only in a wild-type background. It would be good to have the expression in the polo9 background - one would like to know how big the remaining contribution of the endogenous gene is. Has this allele been analyzed molecularly? Is it just a point mutation so that the mRNA level is unchanged?

4. p. 13 and Fig. 4A: It would be good to have an unstable RNA as a control to make sure that the actinomycin D treatment worked. (Actinomycin is not a very stable molecule.)

5. I agree that the explanation of the phenotype by a translational effect is likely to be true. However, the whole story is based on the analysis of transgenic flies, and I do not think the authors excluded a simple difference in RNA levels in the transgenic lines: They show, by fluoresence, that the two delta pA2 lines express less GFP-polo, but I did not see any comparison of the RNA levels in the different transgenic lines. This seems essential.

6. The authors did not find an effect of two miRNAs on polo expression in an overexpression experiment. Can they exclude that the miRNAs were already present at sufficient levels?

7. p. 18, top paragraph: For my taste, there is too much speculation in this section.

Minor points:

p. 4, line 6: I am not sure I understand the sentence correctly, but I believe the comma behind 'Drosophila' should be deleted.

Do not use non-standard abbreviations (APA, p. 5; APF, p. 11).

Fig. 2B is referred to before Fig. 1 (p. 7)

p. 9, line 11 below the heading: I believe the word 'were' should be deleted.

p. 10, lines 3 and 4 from the bottom: '...the insertion sites of the transgene were mapped and shown to have different integration sites....' - this sentence is poorly constructed.

p. 13, line 5 from the bottom: 'The 3' UTR ending at pA1....' - the second part of this sentence is poorly connected.

p. 16, line 4: The term 'elongation rate' would be more appropriate than 'processivity'.

p. 17, line 6: The word 'similarly' is not appropriate, as the sentence describes just the opposite of the preceding one - higher expression upon use of the proximal site.

Referee #3 (Remarks to the Author):

This is an extremely interesting paper that investigates the use of two alternative polyadenylation sequences in the 3'UTR of the mRNA for the Droopshila gene polo. The authors use a mutant form of RNA Polymerase II that results in a lower rate of transcription. This results in preferential use of the proximal polyadenylation site (for whatever reason). They go on to show that a transgene in which the distal site is absent cannot rescue a strong hypomorphic polo mutant. The analysis is consistent with the effect being due to differential translation efficiency of the mRNAs having either proximal or distal poly A addition. The experiments have been carried out extremely carefully and give insight into the requirements for differential use of poly adenylation sites in a system in which translational efficiency is one important means of regulating cell proliferation. I think because there has been so little about the use of alternative polyadenylation sites in a biological context, that this paper will create a lot of interest. Therefore I strongly support the publication of the paper without the need for revision.

1st Revision - authors' response 22 December 2010

Referee #1 (Remarks to the Author):

This paper addresses two distinct questions: 1. Does the rate of transcription elongation affect the choice of polyadenylation site? and 2. is there a biological effect of polyA site choice in the polo gene? The data generally support that the answer to each question is yes. However it is not clear if there was an attempt to link these two observations; is there a biological effect of the change in polo expression in the mutant containing the "slow" polymerase mutant?

The data on the slow polymerase is restricted to a single transcript. To make definitive conclusions that the speed of the polymerase affects polyA site choice some additional alternative polyA sites need to be tested. To make the general conclusions the authors want to make requires at least 5 example of alternatively polydenylated transcripts. transcripts.

In our revised manuscript we have aimed to more closely connect the effect of the slow Pol II on *polo* pA site selection with the simple selective inactivation of each pA site. Thus it is clear that the slow Pol II affects alternative pA site choice both for *polo* and now also, as shown in our new Figure 1F, for other fly pA sites.

However as extensively described our reworked Discussion, the slow Pol II fly phenotype appears less severe then the simple *DpA2* lethal phenotype. We discuss this difference which we feel is likely to be due to (1) significant levels of pA2 *polo* mRNA are still made with the slow Pol II mutant, presumably enough to allow fly viability and the development of a normal abdomen and (2) the fact that slow Pol II will affect many genes.
Please see also answer to referee #2.

Specific comments:

1. The organization of the paper at the start is confusing, since they start by discussing Fig. 2. It would be better to start the paper with Fig. 2, and the diagram of the gene and the two polyA sites. The Northern blot shows they are used about equally under normal conditions. It would be useful to have a Northern also for the C4 mutant, although the PCR in Fig. 2C is convincing.

As suggested, we have inserted a diagram of the *polo* gene in Figure 1A, drawn at scale, also showing ChIP probe positions (question #2 below).

Please see also text in page 9 and 11 for more information regarding the levels of *polo* pA1 and *polo* pA2 transcripts presented by adult flies and 3rd instar larvae.

2. The interpretation of the CHIP data is not clear. In the wild-type there is a dramatic drop after the first polyA site, although the data shows that equal numbers of long and short transcripts are made. It is not at all clear how one can explain that drop. The authors should comment on the size of the DNA fragments obtained after shearing for the chip experiments, and how it compares to the size and separation of the different probes. A scale on Fig. 1A and/or 2A would be helpful. For example, the distance between probes 6 and 7 is likely less than 200 nts, which probably overlaps with the size of the DNA fragments obtained in the CHIP protocol. The "slow" polII gives more polydenylation at site 1, which could lead to subsequent termination before site 2. Thus the CHIP data is the opposite of what one would intuitively expect and the authors need to discuss this.

We accept this reviewers point about our ChIP analysis (Figure 1A). However the fact that we see a clear reduction in Pol II ChIP signal in wild type over the *polo* 3'UTR still argues for a termination effect in wild type. The slow Pol II mutant loses this termination effect and shows overall lower Pol II levels. We feel these results are interesting and must relate to pA site selection. However (as recommended by referee #2) we do not want to over interpret these data other than to demonstrate that slow Pol II does show a significant effect using ChIP analysis.

The fragmentation size of the DNA used in ChIP and how this compares to the size and separation of the different probes are now discussed in the text. A size scale is also included in Figure 1A.
Please see also text in page 9 and 11 for more information regarding the levels of *polo* pA1 and *polo* pA2 transcripts presented by adult flies and 3rd instar larvae.

3. How the two polyA site usage is "quantified" by qPCR is not clear. Any primer used to amplify the short transcript will also amplify the long transcript. If it is done by 3' RACE, then it is certainly qualitative and not quantitative.

We apologize that by mistake this information was not included. To clarify this point we have included information regarding quantification in Materials and Methods plus text clarification (page 9), and re-drawn the Figure 1E. The graph represents total amounts of *polo* transcripts / *polo* pA2 transcripts, quantified by qPCR in adult flies. This was used as an indirect measure of pA1 signal usage. The 3'RACE shown in Figure 1D is qualitative and it was not used for the quantification.

4. near end page 8, When they say that the change in polyA site selection has no effect on expression, do they mean mRNA or protein?. From the rest of the paper, the protein levels should be affected if the short polyA form is the major one, and it doesn't get translated well. The western

blot in supp. Fig. 1B shows no effect on the protein. Since this is unexpected given the change in polyA site usage this data should be included in Fig. 1, rather than in supplemental data.

The Western blot is now included in Figure 1C. Please see text for clarification.
It is true that we do not see a decrease in Polo protein with mutant flies, but this can be explained by the fact that in these flies *polo* pA2 mRNA, which is efficiently translated, is still produced (Figure 1) in contrast to *DpA2* flies. Please see also explanation added to text in Results and Discussion (page 20).

5. The "cryptic" polyA site in Fig. 2C, needs to be documented or removed from the paper. If it really is a small fraction of the transcripts, how do the authors know it isn't present in the wild-type also?

This was now removed from the paper as suggested. See also answer #2 to referee #2.

6. The Western blot shown in Fig. 5C does not support the graph in Fig. 5C. There is reduction in both tubulin and GFP-polo on overexpression of polo and certainly the data don't look like the 5-fold change reported in the graph.

We apologize that by mistake the gel originally shown did not correspond to the graph. The correct Western is now in place in Figure 5C.

Referee #2 (Remarks to the Author):

The authors have examined alternative polyadenylation in the Drosophila gene encoding the polo kinase, an important cell cycle regulator.

They find that a mutant RNA polymerase II with a lower elongation rate leads to an increase in the use of the first of two alternative polyadenylation sites. This supports a first come, first served model. They also show that use of the second site is essential for fly development. RNAs terminating at the second site are translated more efficiently.

The data of this paper are technically mostly convincing. One major flaw is mentioned below.
There is little connection between the part of the paper concerning the pol II mutant and the rest, as the pol II mutant flies do not seem to have a phenotype that could be explained by a decreased use of the second poly(A) site. There is also not too much mechanistic insight in the paper. Why is the longer RNA translated more efficiently?

As discussed in response to referee #1 comments, we have extended our discussion on why the slow Pol II phenotype differs from the *DpA2* phenotype. In detail, the abdominal phenotype observed in *gfp-poloDpA2;polo*^{9} flies is due to the fact that these flies do not express *polo* pA2 mRNA; the *polo* pA1 transcript produced by these flies does not produce enough amounts of protein for the flies to survive the pupa stage (Fig. 3B, C). However, *RpII215* mutants still express *polo* pA2 mRNA (Fig. 1) and therefore these mutants still have enough mRNA to produce the required amount of Polo protein to allow abdominal morphogenesis to take place.

We are indeed doing the experiments to understand how mechanistically *polo* pA2 is more efficiently translated than *polo* pA2. However we feel that such experiments are beyond the scope of this already heavy paper. We have obtained preliminary data suggesting that ELAV/HuR binds to the shorter *polo* pA1 transcripts together with PTB and hnRNPC, and that these may be involved in the mechanism. The present paper is intended to show the biological importance of a widespread phenomenon, alternative polyadenylation, in a living organism. Further mechanistic results will hopefully follow in future publications.

Specific comments:

1. p. 8: I do not see how a reduced elongation rate should reduce the number of pol II molecules on the gene. If anything, an increase would be expected. A decrease could be explained by (i) a lower initiation frequency (ii) an increase in premature termination and (iii) a reduced recovery during

the ChIP procedure. I do not think that the paper depends on an explanation, but none is preferable to one that does not make sense.

We accept this point and so have removed this discussion from the revised manuscript.

2. p. 9: Could the authors give us a rough idea what 'a small proportion of transcripts ending at a cryptic pA site' means? How small?

We have removed the cryptic pA site data as we feel it is quantitatively irrelevant to this paper.

3. p. 10 and Fig. 2B: The expression of the polo transgenes is shown only in a wild-type background. It would be good to have the expression in the polo9 background - one would like to know how big the remaining contribution of the endogenous gene is. Has this allele been analyzed molecularly? Is it just a point mutation so that the mRNA level is unchanged?

No, it is not a point mutation, the *polo*^{9} mutant has a P-element inserted in the 5'UTR of *polo* gene, affecting its transcription units. This mutant has been shown to have almost undetectable levels of Polo protein by Western that are not sufficient for the individuals to develop beyond the 3rd instar larval stage of development (Donaldson et al, 2001). Therefore, we do not expect that *polo*^{9} contribution affect the expression of the different transgenes during the pupa stage. Nonetheless, we now quantified the *polo*^{9} mRNA contribution by qPCR and clarified this point in the text – please see page 11-13 and Fig. 2E. As shown, the mRNA levels contributed by *polo*^{9} (the difference between total *polo* and *gfp-polo* bars in the graph in Fig 2 E) are minor.

4. p. 13 and Fig. 4A: It would be good to have an unstable RNA as a control to make sure that the actinomycin D treatment worked. (Actinomycin is not a very stable molecule.)

We have performed this important control as suggested. Please see Figure 4A and associated text. Happily this valuable suggestion provides a useful positive control to our data.

5. I agree that the explanation of the phenotype by a translational effect is likely to be true. However, the whole story is based on the analysis of transgenic flies, and I do not think the authors excluded a simple difference in RNA levels in the transgenic lines: They show, by fluoresence, that the two delta pA2 lines express less GFP-polo, but I did not see any comparison of the RNA levels in the different transgenic lines. This seems essential.

Detecting the amount of mRNA in histoblasts cells is technically very challenging to do because these cells are very hard to isolate – please see the very small size of histoblasts in Figure 3A. FISH is not quantitative and is also a difficult technique. This problem explains why all 35 papers published on histoblasts in Drosophila only present microscopy work at the protein level. Martin-Blanco (one of the world experts in Drosophila histoblasts) has used fly genetics to obtain histoblast cells labelled with a dye. We have done this and then tried to sort out these cells by FACS. However we failed to obtain reliable RT-qPCR data with this material.

Instead, we have now analyzed the mRNAs produced in the same transgenic pupae as those used in Fig. 3B, by qPCR - please see new data in Fig. 3D and correspondent text. There is no difference between the mRNA levels produced by *DpA2;polo*^{9}*/TM6B* and *gfp-polo;polo*^{9}*/TM6B* pupae (Fig. 3D) but less protein is being produced (Fig. 3B and C). This argues against an effect of low protein production due to reduced mRNA levels.

6. The authors did not find an effect of two miRNAs on polo expression in an overexpression experiment. Can they exclude that the miRNAs were already present at sufficient levels?

Our results on potential miRNA involvement by overexpression technique are only preliminary and not central to this paper. However to address the referee's concern we performed two experiments. To exclude the possibility that endogenous mir-8 and mir-1016 act on *polo* 3'UTR, we did a control experiment, where Luc is followed by SV40 pA and compared with the same vector containing *polo* 3'UTR – shown in Sup Fig 6 B. There is no difference in *luc* mRNA and activity levels for the two plasmids, suggesting that miRNAs are not acting on *polo* 3'UTR. We also quantified the levels of these miRNAs, as suggested by the referee (shown in Sup Fig 6D). dme-mir-1016 is expressed in

very low levels, whilst dme-mir-8 is expressed at higher levels. Nevertheless, the amount of each miRNA produced by overexpression does not correlate with any effect on *polo* 3'UTR expression, indicating that these miRNAs are unlikely to be involved in *polo* silencing.

7. p. 18, top paragraph: For my taste, there is too much speculation in this section.

Deleted part of the text, and adjusted the rest.

Minor points:

p. 4, line 6: I am not sure I understand the sentence correctly, but I believe the comma behind 'Drosophila' should be deleted.

comma behind *Drosophila* was deleted

Do not use non-standard abbreviations (APA, p. 5; APF, p. 11).

APA and APF were substituted in the manuscript for alternative polyadenylation and after pupa formation, respectively.

Fig. 2B is referred to before Fig. 1 (p. 7)

Changed

p. 9, line 11 below the heading: I believe the word 'were' should be deleted.

the verb was moved further down in the sentence.

p. 10, lines 3 and 4 from the bottom: '...the insertion sites of the transgene were mapped and shown to have different integration sites....' - this sentence is poorly constructed.

second part of the sentence starting with "The possibility…." was deleted.

p. 13, line 5 from the bottom: 'The 3' UTR ending at pA1....' - the second part of this sentence is poorly connected.

second part of the sentence starting with "The 3' end…" was deleted

p. 16, line 4: The term 'elongation rate' would be more appropriate than 'processivity'.

"processivity" was changed to elongation rate

p. 17, line 6: The word 'similarly' is not appropriate, as the sentence describes just the opposite of the preceding one - higher expression upon use of the proximal site.

"similarly" was deleted from the beginning of the sentence.

The following minor modifications were also made:

- mention to a recent relevant work in the field and more recent references added.
- '*Oligos used in the study'* as well as '*miR quantification'* methods were now moved to Supplementary information, due to space constraints.

Referee #3 (Remarks to the Author):

This is an extremely interesting paper that investigates the use of two alternative polyadenylation sequences in the 3'UTR of the mRNA for the Droopshila gene polo. The authors use a mutant form

of RNA Polymerase II that results in a lower rate of transcription. This results in preferential use of the proximal polyadenylation site (for whatever reason). They go on to show that a transgene in which the distal site is absent cannot rescue a strong hypomorphic polo mutant. The analysis is consistent with the effect being due to differential translation efficiency of the mRNAs having either proximal or distal poly A addition. The experiments have been carried out extremely carefully and give insight into the requirements for differential use of poly adenylation sites in a system in which translational efficiency is one important means of regulating cell proliferation. I think because there has been so little about the use of alternative polyadenylation sites in a biological context, that this paper will create a lot of interest. Therefore I strongly support the publication of the paper without the need for revision.

Finally we are grateful to our referees for their insightful reviews of our manuscript. By following their suggestions we have been able to generate a much tighter and hopefully more lucid account of *polo* alternative polyA site selection.

3rd Editorial Decision 27 January 2011

As you will be aware after the original round of review of your manuscript we were in a situation where we had two contrasting recommendations, one referee (#2) recommend that the manuscript should not be published in The EMBO Journal, while another referee (#3) found the study suitable for publication. Referee #2 was more positive but required that the two parts of the study describing the role of polymerase kinetics on pA usage and the requirement of the long isoform for development to be connected and further insight into the molecular basis of the phenotype.

Given that referee #2 and #3 provided strong albeit conflicting recommendations, I sent the revised manuscript to referee #1 for evaluation. I have now received his/her report and unfortunately, they find that the study is not suitable for publication in The EMBO Journal. The main reason for this is the remaining disconnect between the two different aspects of the study, the slow polymerase while increasing the efficiency of pA1 usage has no functional effect on polo levels. In addition, while it is clear that the long form of polo is required to rescue the developmental defects in the polo hyopmorph, the molecular basis of this is not clear. As a result while the manuscript has been extended the referee finds that it describes two separate phenomena that should be published separately and unfortunately neither is sufficiently developed for The EMBO Journal. Furthermore, it remains unclear if this could be resolved even during another major round of revision. Therefore, given that the journal only allows a single round of revision and that two referees now recommend against publication, I find that that we cannot proceed further with the manuscript at The EMBO Journal. I would like to thank you for submitting your manuscript to the EMBO Journal for consideration and I am truly sorry that we cannot be more positive on this occasion.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The revised paper has addressed many of the concerns. However, there are still really two distinct stories: 1. the slow polymerase affects polyA site choice and 2. the two different polo polyadenylated mRNAs are essential. There is really no connection between these two since the slow polymerase has no effect on the polo mutant. There are still substantial overstatements in the paper. For example, the next to last sentence of the introduction says "Consequently we demonstrate the need to precisely select the correct polyA signal for proper protein level production, cell

proliferation and viability in the living organism". Actually they have not done that. The change in relative amounts of polyA site selection as a result of the slow polymerase has no biological effect and no effect on polo protein levels. This is true even though there are changes in relative polyA site selection for a number of mRNAs.

The authors show that there is likely a regulatory element (positive effect on translation)in the pA2 UTR and it should be possible to define the element given the relatively small increase in length of the pA2 3' UTR. That would add substantially to the paper.

Specific comments:

1. Fig. 1D would be helped by showing a diagram of the two transcripts and the primers they used. Presumably they used one primer on dT and then two upstream primers one for each polyA site. Indicating the size of the PCR products would also be useful since that helps judge the relative amounts of each product.

2. As I read Fig. 1E, there is 83% pA1 usage normally and in the polymerase mutant there is 95% pA1. I actually don't understand how this relatively small absolute change in pA1 can be responsible for the large difference in CHIP over site 7 in Fig. 1A. A more likely explanation is that the change in the mutant polymerase is what causes the change in CHIP. Perhaps the mutant polymerase is defective in termination or pauses for a long time at the termination site. Checking what happens on another gene might be helpful.

3. A general comment about the CHIP. The size of the sheared fragments is about 200 nts and the difference between adjacent fragments is smaller than that. This means that there has to some overlap in signal (i.e. one cannot completely separate the signals from adjacent sequences). Given the gene they are looking at there is no way to avoid this problem, but they should be cautious in their interpretation.

4. What the authors do show convincingly is that expression of only pA1 mRNA is not sufficient to rescue the polo mutant, while expression of the intact gene (expressing both mRNAs) or the pA2 mRNA results in rescue of the mutant. This is a very interesting result given the relatively small amount of the pA2 mRNA expressed normally. They could look to see if there is a difference in the amount of pA2 normally expressed in histoblasts by in situ hybridization using a probe specific for pA2 mRNA.

5. The authors propose an autoregulatory loop where polo can feedback on its own expression by changing the polyadenylation site. Two experiments are shown. The first one shows a change in polyadenylation site levels. If we assume from Fig. 1E, that the base usage is 83% pA1, the 2.5 fold change reported changes the usage to 93% pA1. The second experiment shows down-regulation of GFP-polo protein by overexpressing polo. It is very common to see degradation of the tagged proteins in the presence of excess wild-type protein, since often the tagged protein is less efficient in forming complexes required for function compared with the wild-type protein. Thus the autoregulation model, while potentially attractive, is certainly not proven.
Minor comments:

Pg 6, next to last line: they mean "exon" not "axon".

Rebuttal 30 January 2011

We are dismayed by your decision on our revised EMBO J. manuscript. Whilst we agreed with most of the concerns raised by the referees in the first review, I now feel that referee 1 has treated us quite unfairly in his/her review of our revised ms. Since we completed most of the experiments that were asked for in our revised ms we anticipated that referee 1 would now be happy with the paper. The reasons for our disquiet are as follows.

1 He/She does not appear to have read our "Response to the reviewers" document where the questions now raised were already answered!

3 He/She now raises new questions (1 and 5), that were not mentioned in the first review. It is unfair to "move the goal posts" like this.

4 He/She does not appear to be very familiar with the model system (drosophila) we use in this study. We wonder whether this reviewer is really the same person who reviewed the ms the first time round (Reviewer 1) or rather a postdoc in the lab? Our response to this review of our revised ms is as follows

However, there are still really two distinct stories: 1. the slow polymerase affects polyA site choice and 2. the two different polo polyadenylated mRNAs are essential. There is really no connection between these two since the slow polymerase has no effect on the polo mutant.

This point was fully discussed in our revised ms Discussion and in our responses to referees 1 and 2. The slow polymerase has no effect on Polo levels because the mutation in the Pol II is "leaky" and still uses pA2. The "slow" polymerase was shown by D. Price and A. Greenleaf's labs to have an elongation rate decreased by 50%. We show that it preferentially uses the most proximal poly(A) signal in the 3'UTR. We did the experiments suggested by this referee, and showed the same effect for other 6 new genes. Because the slow RNA Pol II still utilizes the distal signal to some extent it makes some longer transcript (Figure 1D and E). Presumably in the case of Polo this transcript provides sufficient protein for flies to develop normally. This is why we do not see a strong phenotype in slow Pol II mutant flies. Nevertheless, we feel that the two stories are closely connected, because they both deal with the importance of correctly choosing an alternative poly(A) site in a living organism, something that was never done before. Moreover, in view of the publications from Bartel, Sharp and Burge's labs showing a relationship between alternative polyadenylation, cell differentiation and cancer, we feel that our findings, on the role of Pol II kinetics in poly(A) site selection and polo pA2 deletion are a clear advance in the field.Our paper describes both important biological and mechanistic data on a really widespread phenomenon, alternative polyadenylation.

Recent genomewide analysis does not in general provide mechanistic information so that our data is novel as it shows the clear biological importance of alternative poly(A) site selection.

There are still substantial overstatements in the paper. For example, the next to last sentence of the introduction says "Consequently we demonstrate the need to precisely select the correct polyA signal for proper protein level production, cell proliferation and viability in the living organism". Actually they have not done that.

We were surprised to see this comment now, as we were not asked to change this sentence in the first review. As it can be easily understood by reading the text, this sentence relates to the previous ones regarding polo delta pA2 flies: Our revised text "Moreover we show that in flies carrying a deletion of the polo pA2 signal, Polo is translated at low levels and as a result precursor cells of the abdominal epidermis do not proliferate during metamorphosis. This causes a defect in abdomen development and lethality. Consequently we demonstrate the need to precisely select the correct pA signal for proper protein levels production, cell proliferation and viability in a living organism." We disagree with the reviewer on this point. We have shown that flies without polo pA2 die, as they are forced to produce only polopA1 transcript by pA1 signal usage (Figure 2B and D and Table I). We also show that polopA1 transcript produces lower protein levels in histoblasts (Figure 3B) because it's translated less efficiently (Figure 4 B). Thus, by using only pA1, the flies produce only low levels of protein resulting in a lethal developmental defect. However, we do accept that the sentence could be further clarified with the inclusion of " polo polyA2 ", and we are happy to do this, eg:"...the need to precisely select the correct polo polyA2 signal for proper protein level production, cell proliferation and viability in the living organism."

The authors show that there is likely a regulatory element (positive effect on translation)in the pA2 UTR and it should be possible to define the element given the relatively small increase in length of the pA2 3' UTR. That would add substantially to the paper.

You will appreciate that a scientific story is never finished. The paper already has 6 figures, 1 table and 6 supplementary figures. Moreover, the paper deals with RNA polymerase II kinetics and the

biological effect of alternative poly(A) site selection. We feel that is inappropriate to include yet more figures about the factors that may be involved in regulating polo expression. We did give the answer below to referee 2: "We are indeed doing the experiments to understand how mechanistically polo pA2 is more efficiently translated than polo pA2. However we feel that such experiments are beyond the scope of this already heavy paper. We have obtained preliminary data suggesting that ELAV/HuR binds to the shorter polo pA1 transcripts together with PTB and hnRNPC, and that these may be involved in the mechanism. The present paper is intended to show the biological importance of a widespread phenomenon, alternative polyadenylation, in a living organism. Further mechanistic results will hopefully follow in future publications."

Specific comments

1. Fig. 1D would be helped by showing a diagram of the two transcripts and the primers they used. Presumably they used one primer on dT and then two upstream primers one for each polyA site. Indicating the size of the PCR products would also be useful since that helps judge the relative amounts of each product.

This is a standard 3'RACE experiment so we used only one primer upstream of pA1 for both samples.This figure is illustrative only, as 3'RACE is not quantitative. We also show quantification by realtime analysis in Fig. 1 E.

2. As I read Fig. 1E, there is 83% pA1 usage normally and in the polymerase mutant there is 95% pA1. I actually don't understand how this relatively small absolute change in pA1 can be responsible for the large difference in CHIP over site 7 in Fig. 1A. A more likely explanation is that the change in the mutant polymerase is what causes the change in CHIP. Perhaps the mutant polymerase is defective in termination or pauses for a long time at the termination site. Checking what happens on another gene might be helpful.

3. A general comment about the CHIP. The size of the sheared fragments is about 200 nts and the difference between adjacent fragments is smaller than that. This means that there has to some overlap in signal (i.e. one cannot completely separate the signals from adjacent sequences). Given the gene they are looking at there is no way to avoid this problem, but they should be cautious in their interpretation.

We think this comment is unfair as we removed parts of the text in order to not overinterpret the ChIP data. The argument the referee uses now, "Perhaps the mutant polymerase is defective in termination or pauses for a long time at the termination site" is similar to the sentence we had written in our first version of the manuscript and that we removed in the revised version. Moreover, we addressed the concerns presented by referees #1 and #2 in our letter of response to the referees: "Presumably the slower Pol II elongation rate in this mutant reduces overall Pol II residency across the gene."We answered referee #1 point on this issue previously in our response letter:"We accept this reviewers point about our ChIP analysis (Figure 1A). However the fact that we see a clear reduction in Pol II ChIP signal in wild type over the polo 3'UTR still argues for a termination effect in wild type. The slow Pol II mutant loses this termination effect and shows overall lower Pol II levels. We feel these results are interesting and must relate to pA site selection. However (as recommended by referee #2) we do not want to over interpret these data other than to demonstrate that slow Pol II does show a significant effect using ChIP analysis."

4. What the authors do show convincingly is that expression of only pA1 mRNA is not sufficient to rescue the polo mutant, while expression of the intact gene (expressing both mRNAs) or the pA2 mRNA results in rescue of the mutant. This is a very interesting result given the relatively small amount of the pA2 mRNA expressed normally. They could look to see if there is a difference in the amount of pA2 normally expressed in histoblasts by in situ hybridization using a probe specific for pA2 mRNA.

FISH is not quantitative!We answered this question before, to referee 2: "Detecting the amount of mRNA in histoblasts cells is technically very challenging to do because these cells are very hard to isolate - please see the very small size of histoblasts in Figure 3A. FISH is not quantitative and is also a difficult technique. This problem explains why all 35 papers published on histoblasts in Drosophila only present microscopy work at the protein level. Martin Blanco (one of the world

experts in Drosophila histoblasts) has used fly genetics to obtain histoblast cells labelled with a dye. We have done this and then tried to sort out these cells by FACS. However we failed to obtain reliable RTqPCR data with this material. Instead, we have now analyzed the mRNAs produced in the same transgenic pupae as those used in Fig. 3B, by qPCR please see new data in Fig. 3D and corresponding text. There is no difference between the mRNA levels produced by pA2;polo9/TM6B and gfppolo;polo9/TM6B pupae (Fig. 3D) but less protein is being produced (Fig. 3B and C). This argues against an effect of low protein production due to reduced mRNA levels."

5. The authors propose an autoregulatory loop where polo can feedback on its own expression by changing the polyadenylation site. Two experiments are shown. The first one shows a change in polyadenylation site levels. If we assume from Fig. 1E, that the base usage is 83% pA1, the 2.5 fold change reported changes the usage to 93% pA1. The second experiment shows downregulation of GFPpolo protein by overexpressing polo. It is very common to see degradation of the tagged proteins in the presence of excess wildtype protein, since often the tagged protein is less efficient in forming complexes required for function compared with the wildtype protein. Thus the autoregulation model, while potentially attractive, is certainly not proven.

The referee claims that the difference in pA site usage determined in flies overexpressing Polo is similar to the one shown by the RNA pol II mutant flies. We don't understand why he says this. In this set of data we are comparing flies overexpressing Polo with the wild type strain. Regarding the method used, it was previously shown to work correctly and published at the EMBO J (Martins T et al, 2009, EMBO J 28: 234247) and in Development (Mirouse V et al. (2006) Development. 133: 40054013). It's a perfectly well established method.Nevertheless we are happy to remove the polo autoregulatory model from figure 6, if this were thought to improve the paper.Given all this, we feel that our revised version of the manuscript has not been adequately reviewed by referee 1. We ask you to reconsider your decision. Perhaps an independent referee could look at our manuscript.

Additional correspondence (editor) 21 February 2011

I spent Friday afternoon reading both versions of your manuscript and the referee reports, correspondence etc. I also have discussed it with the Chief Editor today. I have a few questions that I would like to ask you, I will write a brief letter while I am traveling. I will send them to you as soon as I have email contact on Wednesday.

Yours sincerely,

Editor
The EMBO Journal

Additional corresopndence (editor) 23 February 2011

I would be grateful to hear your response to the below questions. Is there evidence for a switch in alternative polyadenylation during development, does the levels of the long isoform increase? Is there any evidence for a function for the short isoform? With the polymerase mutant, I agree that a potential reason for not seeing a phenotype may be due the fact that relatively high levels of the long isoform persist. However does this mean that the effect of polymerase kinetics does not play an important biological role during development, are there stronger polymerase alleles (although this would probably result in many other defects)? This refers back to point 1, if polo alternative polyadenylation site usage occurs during development,Is this the first example of polymerase kinetics regulating polyadenyation site choice?

Yours sincerely,

Editor

The EMBO Journal

Additional correspondence (author) 24 February 2011

Thank you for allowing us the opportunity to address your specific questions.

Editor: Is there any evidence for a function for the short isoform?

We clearly show that there is an autoregulatory function for the short isofom:a) When polo is overexpressed by 23 fold in the living fly (Fig. 5A), there is a switch in polo pA site usage with pA1 now used 2.5 fold more efficiently (Fig. 5B). This results in the formation of higher amounts of the shorter polo mRNA that is translated into 75% less protein (Fig. 5C). Thus, although the shorter mRNA does not produce a different protein it acts to autoregulate the levels of Polo protein (Figure 6B).b) We also show that transgenic female flies without pA1 in a null background display small but clear malformations in the abdomen, in comparison with the wild type (Figure 2D, compare panels 5 and 6). Furthermore female flies without pA1 are sterile. Both of these phenotypes argue that the shorter isoform does have a physiological function. We suggest that this function is associated with Polo autoregulation.

Editor: With the polymerase mutant, I agree that a potential reason for not seeing a phenotype may be due the fact that relatively high levels of the long isoform persist. However does this mean that the effect of polymerase kinetics does not play an important biological role during development, are there stronger polymerase alleles (although this would probably result in many other defects)? This refers back to point 1, if polo alternative polyadenylation site usage occurs during development.

Pol II kinetics are known to play an important biological role during Fly development, because stronger alleles also present much stronger developmental defects (Ubx) and some of these alleles die as embryos. Unfortunately these strains were not available to us. However the strain we used in this paper is the only one where the transcription rate has been thoroughly characterized by biochemical methods. We did consult Arno Greenleaf, who described these early biochemical studies and showed that the point mutation in the Pol II causes a 50% decrease in its transcription rate (Chen et al, 1996).Importantly this 50% decrease in the transcription rate is sufficient to cause a pA switch in at least six genes in vivo (including a 4fold increase in polo pA1 usage and a 3 fold increase in CG6024 pA1 usage, in comparison to wild type flies), which argues that kinetics plays an important role in alternative poladenylation. Indeed to our knowledge this is the first time that transcription elongation rate has been correlated with altenative pA site usage. In view of the very wide spread occurrence of alternative polyadenylation this is an important advance in the field.We have no evidence for polo alternative polyadenylation site usage during fly development. In early studies (Llamazares et al, 1991) polo was analysed by Northern blot at some stages of development and it seemed that both isoforms were present.However according to a recent study on alternatively polyadenylated genes (Ji et al, 2009), distal pA is predominantely used during mouse development. This nicely fits in with our results: at the metamorphosis polo pA2 would be more used than pA1, producing more Polo protein, which is necessary for cell divisions. That is exactly the developmental stage where we see defects when pA2 is completely abolished.

Editor: Is this the first example of polymerase kinetics regulating polyadenyation site choice?

As already stated this is the first example! (But given the current interest in alternative polyadenylation in development, cancer, differentiation, it will not be if we don't publish soon!) In detail our study is the first in vivo example of how a 50% decrease in Pol II transcription rate can affect polyadenylation site choice in at least 7 endogenous different genes, in the 3'UTR, independently of alternative splicing. These results clearly demonstrate that Pol II kinetics regulates polyadenylation site choice.

I hope our response to your questions reassures you that this paper does provide important new data on the mechanism and biological significance of alternative polyadenylation. As such this study will be of general interest in Molecular Biology.

References:Chen Y, Chafin D, Price DH, Greenleaf AL (1996) Drosophila RNA polymerase II mutants that affect transcription elongation. J Biol Chem 271: 59935999Ji Z, Lee JY, Pan Z, Jiang B, Tian B (2009) Progressive lengthening of 3' untranslated regions of mRNAs by alternative polyadenylation during mouse embryonic development. Proc Natl Acad Sci U S A 106: 70287033Llamazares S, Moreira A, Tavares A, Girdham C, Spruce BA, Gonzalez C, Karess RE, Glover DM, Sunkel CE (1991) polo encodes a protein kinase homolog required for mitosis in Drosophila. Genes & Development 5: 21532165

Appeal 28 March 2011

Letter of response to the referees' comments

Referee #1 (Remarks to the Author):

This paper addresses two distinct questions: 1. Does the rate of transcription elongation affect the choice of polyadenylation site? and 2. is there a biological effect of polyA site choice in the polo gene? The data generally support that the answer to each question is yes. However it is not clear if there was an attempt to link these two observations; is there a biological effect of the change in polo expression in the mutant containing the "slow" polymerase mutant?

The data on the slow polymerase is restricted to a single transcript. To make definitive conclusions that the speed of the polymerase affects polyA site choice some additional alternative polyA sites need to be tested. To make the general conclusions the authors want to make requires at least 5 example of alternatively polydenylated transcripts. transcripts.

In our revised manuscript we have aimed to more closely connect the effect of the slow Pol II on polo pA site selection with the simple selective inactivation of each pA site. Thus it is clear that the slow Pol II affects alternative pA site choice both for polo and now also, as shown in our new Figure 1F, for other fly pA sites.

However as extensively described our reworked Discussion, the slow Pol II fly phenotype appears less severe then the simple DpA2 lethal phenotype. We discuss this difference which we feel is likely to be due to (1) significant levels of pA2 polo mRNA are still made with the slow Pol II mutant, presumably enough to allow fly viability and the development of a normal abdomen and (2) the fact that slow Pol II will affect many genes.

Please see also answer to referee #2.

Specific comments:

1. The organization of the paper at the start is confusing, since they start by discussing Fig. 2. It would be better to start the paper with Fig. 2, and the diagram of the gene and the two polyA sites. The Northern blot shows they are used about equally under normal conditions. It would be useful to have a Northern also for the C4 mutant, although the PCR in Fig. 2C is convincing.

As suggested, we have inserted a diagram of the polo gene in Figure 1A, drawn at scale, also showing ChIP probe positions (question #2 below).

Please see also text in page 9 and 11 for more information regarding the levels of polo pA1 and polo pA2 transcripts presented by adult flies and 3rd instar larvae.

2. The interpretation of the CHIP data is not clear. In the wild-type there is a dramatic drop after the first polyA site, although the data shows that equal numbers of long and short transcripts are made. It is not at all clear how one can explain that drop. The authors should comment on the size of the DNA fragments obtained after shearing for the chip experiments, and how it compares to the size and separation of the different probes. A scale on Fig. 1A and/or 2A would be helpful. For example, the distance between probes 6 and 7 is likely less than 200 nts, which probably overlaps with the size of the DNA fragments obtained in the CHIP protocol. The "slow" polII gives more

polydenylation at site 1, which could lead to subsequent termination before site 2. Thus the CHIP data is the opposite of what one would intuitively expect and the authors need to discuss this.

We accept this reviewers point about our ChIP analysis (Figure 1A). However the fact that we see a clear reduction in Pol II ChIP signal in wild type over the polo 3'UTR still argues for a termination effect in wild type. The slow Pol II mutant loses this termination effect and shows overall lower Pol II levels. We feel these results are interesting and must relate to pA site selection. However (as recommended by referee #2) we do not want to over interpret these data other than to demonstrate that slow Pol II does show a significant effect using ChIP analysis.

The fragmentation size of the DNA used in ChIP and how this compares to the size and separation of the different probes are now discussed in the text. A size scale is also included in Figure 1A. Please see also text in page 9 and 11 for more information regarding the levels of polo pA1 and polo pA2 transcripts presented by adult flies and 3rd instar larvae.

3. How the two polyA site usage is "quantified" by qPCR is not clear. Any primer used to amplify the short transcript will also amplify the long transcript. If it is done by 3' RACE, then it is certainly qualitative and not quantitative.

We apologize that by mistake this information was not included. To clarify this point we have included information regarding quantification in Materials and Methods plus text clarification (page 9), and re-drawn the Figure 1E. The graph represents total amounts of polo transcripts / polo pA2 transcripts, quantified by qPCR in adult flies. This was used as an indirect measure of pA1 signal usage. The 3'RACE shown in Figure 1D is qualitative and it was not used for the quantification.

4. near end page 8, When they say that the change in polyA site selection has no effect on expression, do they mean mRNA or protein?. From the rest of the paper, the protein levels should be affected if the short polyA form is the major one, and it doesn't get translated well. The western blot in supp. Fig. 1B shows no effect on the protein. Since this is unexpected given the change in polyA site usage this data should be included in Fig. 1, rather than in supplemental data.

The Western blot is now included in Figure 1C. Please see text for clarification.
It is true that we do not see a decrease in Polo protein with mutant flies, but this can be explained by the fact that in these flies polo pA2 mRNA, which is efficiently translated, is still produced (Figure 1) in contrast to DpA2 flies. Please see also explanation added to text in Results and Discussion (page 20).

5. The "cryptic" polyA site in Fig. 2C, needs to be documented or removed from the paper. If it really is a small fraction of the transcripts, how do the authors know it isn't present in the wild-type also?

This was now removed from the paper as suggested. See also answer #2 to referee #2.

6. The Western blot shown in Fig. 5C does not support the graph in Fig. 5C. There is reduction in both tubulin and GFP-polo on overexpression of polo and certainly the data don't look like the 5-fold change reported in the graph.

We apologize that by mistake the gel originally shown did not correspond to the graph. The correct Western is now in place in Figure 5C.

Referee #2 (Remarks to the Author):

The authors have examined alternative polyadenylation in the Drosophila gene encoding the polo kinase, an important cell cycle regulator.

They find that a mutant RNA polymerase II with a lower elongation rate leads to an increase in the use of the first of two alternative polyadenylation sites. This supports a first come, first served

model. They also show that use of the second site is essential for fly development. RNAs terminating at the second site are translated more efficiently.

The data of this paper are technically mostly convincing. One major flaw is mentioned below. There is little connection between the part of the paper concerning the pol II mutant and the rest, as the pol II mutant flies do not seem to have a phenotype that could be explained by a decreased use of the second poly(A) site. There is also not too much mechanistic insight in the paper. Why is the longer RNA translated more efficiently?

As discussed in response to referee #1 comments, we have extended our discussion on why the slow Pol II phenotype differs from the DpA2 phenotype. In detail, the abdominal phenotype observed in gfp-poloDpA2;polo9 flies is due to the fact that these flies do not express polo pA2 mRNA; the polo pA1 transcript produced by these flies does not produce enough amounts of protein for the flies to survive the pupa stage (Fig. 3B, C). However, RpII215 mutants still express polo pA2 mRNA (Fig. 1) and therefore these mutants still have enough mRNA to produce the required amount of Polo protein to allow abdominal morphogenesis to take place.

We are indeed doing the experiments to understand how mechanistically polo pA2 is more efficiently translated than polo pA2. However we feel that such experiments are beyond the scope of this already heavy paper. We have obtained preliminary data suggesting that ELAV/HuR binds to the shorter polo pA1 transcripts together with PTB and hnRNPC, and that these may be involved in the mechanism. The present paper is intended to show the biological importance of a widespread phenomenon, alternative polyadenylation, in a living organism. Further mechanistic results will hopefully follow in future publications.

Specific comments:

1. p. 8: I do not see how a reduced elongation rate should reduce the number of pol II molecules on the gene. If anything, an increase would be expected. A decrease could be explained by (i) a lower initiation frequency (ii) an increase in premature termination and (iii) a reduced recovery during the ChIP procedure. I do not think that the paper depends on an explanation, but none is preferable to one that does not make sense.

We accept this point and so have removed this discussion from the revised manuscript.

2. p. 9: Could the authors give us a rough idea what 'a small proportion of transcripts ending at a cryptic pA site' means? How small?

We have removed the cryptic pA site data as we feel it is quantitatively irrelevant to this paper.

3. p. 10 and Fig. 2B: The expression of the polo transgenes is shown only in a wild-type background. It would be good to have the expression in the polo9 background - one would like to know how big the remaining contribution of the endogenous gene is. Has this allele been analyzed molecularly? Is it just a point mutation so that the mRNA level is unchanged?

No, it is not a point mutation, the polo9 mutant has a P-element inserted in the 5'UTR of polo gene, affecting its transcription units. This mutant has been shown to have almost undetectable levels of Polo protein by Western that are not sufficient for the individuals to develop beyond the 3rd instar larval stage of development (Donaldson et al, 2001). Therefore, we do not expect that polo9 contribution affect the expression of the different transgenes during the pupa stage. Nonetheless, we now quantified the polo9 mRNA contribution by qPCR and clarified this point in the text - please see page 11-13 and Fig. 2E. As shown, the mRNA levels contributed by polo9 (the difference between total polo and gfp-polo bars in the graph in Fig 2 E) are minor.

4. p. 13 and Fig. 4A: It would be good to have an unstable RNA as a control to make sure that the actinomycin D treatment worked. (Actinomycin is not a very stable molecule.)

We have performed this important control as suggested. Please see Figure 4A and associated text. Happily this valuable suggestion provides a useful positive control to our data.

5. I agree that the explanation of the phenotype by a translational effect is likely to be true. However, the whole story is based on the analysis of transgenic flies, and I do not think the authors excluded a simple difference in RNA levels in the transgenic lines: They show, by fluoresence, that the two delta pA2 lines express less GFP-polo, but I did not see any comparison of the RNA levels in the different transgenic lines. This seems essential.

Detecting the amount of mRNA in histoblasts cells is technically very challenging to do because these cells are very hard to isolate - please see the very small size of histoblasts in Figure 3A. FISH is not quantitative and is also a difficult technique. This problem explains why all 35 papers published on histoblasts in Drosophila only present microscopy work at the protein level. Martin-Blanco (one of the world experts in Drosophila histoblasts) has used fly genetics to obtain histoblast cells labelled with a dye. We have done this and then tried to sort out these cells by FACS. However we failed to obtain reliable RT-qPCR data with this material.

Instead, we have now analyzed the mRNAs produced in the same transgenic pupae as those used in Fig. 3B, by qPCR - please see new data in Fig. 3D and correspondent text. There is no difference between the mRNA levels produced by DpA2;polo9/TM6B and gfp-polo;polo9/TM6B pupae (Fig. 3D) but less protein is being produced (Fig. 3B and C). This argues against an effect of low protein production due to reduced mRNA levels.

6. The authors did not find an effect of two miRNAs on polo expression in an overexpression experiment. Can they exclude that the miRNAs were already present at sufficient levels?

Our results on potential miRNA involvement by overexpression technique are only preliminary and not central to this paper. However to address the referee's concern we performed two experiments. To exclude the possibility that endogenous mir-8 and mir-1016 act on polo 3'UTR, we did a control experiment, where Luc is followed by SV40 pA and compared with the same vector containing polo 3'UTR - shown in Sup Fig 6 B. There is no difference in luc mRNA and activity levels for the two plasmids, suggesting that miRNAs are not acting on polo 3'UTR. We also quantified the levels of these miRNAs, as suggested by the referee (shown in Sup Fig 6D). dme-mir-1016 is expressed in very low levels, whilst dme-mir-8 is expressed at higher levels. Nevertheless, the amount of each miRNA produced by overexpression does not correlate with any effect on polo 3'UTR expression, indicating that these miRNAs are unlikely to be involved in polo silencing.

7. p. 18, top paragraph: For my taste, there is too much speculation in this section.

Deleted part of the text, and adjusted the rest.

Minor points:

p. 4, line 6: I am not sure I understand the sentence correctly, but I believe the comma behind 'Drosophila' should be deleted.

comma behind Drosophila was deleted

Do not use non-standard abbreviations (APA, p. 5; APF, p. 11).

APA and APF were substituted in the manuscript for alternative polyadenylation and after pupa formation, respectively.

Fig. 2B is referred to before Fig. 1 (p. 7)

Changed

p. 9, line 11 below the heading: I believe the word 'were' should be deleted.

the verb was moved further down in the sentence.

p. 10, lines 3 and 4 from the bottom: '...the insertion sites of the transgene were mapped and shown to have different integration sites....' - this sentence is poorly constructed.

second part of the sentence starting with “The possibility....” was deleted.

p. 13, line 5 from the bottom: 'The 3' UTR ending at pA1....' - the second part of this sentence is poorly connected.

second part of the sentence starting with “The 3’ end...” was deleted

p. 16, line 4: The term 'elongation rate' would be more appropriate than 'processivity'.

“processivity” was changed to elongation rate

p. 17, line 6: The word 'similarly' is not appropriate, as the sentence describes just the opposite of the preceding one - higher expression upon use of the proximal site.

“similarly” was deleted from the beginning of the sentence.

The following minor modifications were also made:
- mention to a recent relevant work in the field and more recent references added.
- ‘Oligos used in the study’ as well as ‘miR quantification’ methods were now moved to Supplementary information, due to space constraints.

Referee #3 (Remarks to the Author):

This is an extremely interesting paper that investigates the use of two alternative polyadenylation sequences in the 3'UTR of the mRNA for the Droopshila gene polo. The authors use a mutant form of RNA Polymerase II that results in a lower rate of transcription. This results in preferential use of the proximal polyadenylation site (for whatever reason). They go on to show that a transgene in which the distal site is absent cannot rescue a strong hypomorphic polo mutant. The analysis is consistent with the effect being due to differential translation efficiency of the mRNAs having either proximal or distal poly A addition. The experiments have been carried out extremely carefully and give insight into the requirements for differential use of poly adenylation sites in a system in which translational efficiency is one important means of regulating cell proliferation. I think because there has been so little about the use of alternative polyadenylation sites in a biological context, that this paper will create a lot of interest. Therefore I strongly support the publication of the paper without the need for revision.

Finally we are grateful to our referees for their insightful reviews of our manuscript. By following their suggestions we have been able to generate a much tighter and hopefully more lucid account of polo alternative polyA site selection.

4th Editorial Decision 28 March 2011

Thank you for your cooperation in helping me reach an editorial decision on your manuscript. I have given all the correspondence a great deal of thought and I have also discussed it with my editorial colleagues including the chief editor. After a lot of deliberation I have decided to overturn the original decision and accept the study for publication in The EMBO Journal. I agree that there are some concerns remaining, including how important is the contribution of the polymerase kinetics to poly(A) site choice in vivo, given that little change in protein levels is seen in the polymerase mutant. It is also unclear what is the exact role of the short isoform although a role in the autoregulatory loop is interesting. However, I also agree that the decision of referee #1 after the revision is a bit harsh given that in the first round of review they asked to move the data showing the effect of the slow polymerase on Polo protein levels to the main manuscript from the supplementary material. Rejecting the manuscript based on a lack of change in protein levels may be unfair. Taking everything into consideration, I have decided that although this is the most borderline of cases, there is sufficient new insight into poly(A) site choice and isoform function for the manuscript to be

further considered for publication in The EMBO Journal. I therefore would ask you to incorporate text changes into the manuscript to address the remaining concerns of referee #1 before accepting the manuscript for publication in The EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

4th Revision - authors' response 06 April 2011

Thank you for your email. We have now made the text changes you asked for in order to answer the referee #1 remaining concerns (shown in blue in the manuscript).

Specifically:

- We changed the text throughout the manuscript to avoid overstatements, in particular in pg. 7, pg. 18, pg. 23 and in the legend of Fig. 6.

- We included text mentioning the possible presence of regulatory elements in the 3'UTRs - pg. 22

Answering specific comments from referee #1:

1. A diagram is now included in Fig. 1 D.

2. and 3. We had removed parts of the text in our revised MS in order not to over-interpret the ChIP data and have now added referee #1 comment in pg 9.

4. pg.15 - text added to clarify the levels of polo pA2 mRNA produced. As in situ hybridization is not quantitative, we analyzed the mRNAs by RT-qPCR (please see MS).

5. changed the text in pg 18 and legend of Fig. 6 and included text clarifications regarding the system used in pg 17.

Pg 6, next to last line: they mean "exon" not "axon".

- This error was due to the "auto-correct" function in the text processor. It is now corrected throughout the text.

To further clarify the MS, we also incorporate text changes in:

- pg 19 - to highlight the role of PolII kinetics in APA
- pg 21 - to clarify the difference shown by Rpl215 and DpA2 flies regarding polo pA2 mRNA and protein production, and the effect in development.

We are grateful to you and to our referees for the insightful reviews we have received. By following these suggestions we have been able to further clarify our data and improve our manuscript.

5th Editorial Decision 08 April 2011

I have looked through your revised manuscript and I am satisfied that you have addressed al the concerns as we discussed. I am happy to accept the manuscript for publication in The EMBO Journal, you will receive the official acceptance letter early next week.

Yours sincerely,

Editor
The EMBO Journal

