Role of a versatile peptide motif controlling Hox nuclear export and autophagy in the *Drosophila* fat body

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Original submission

First decision letter

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MS TITLE: Role of a versatile peptide motif in controlling Hox nuclear export and autophagy in the Drosophila fat body

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

I have now received comments on your manuscript from three experts. As you will see, all thought that the work was potentially quite interesting and significant but all also raised a number of concerns that prevent me from accepting the paper. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The study by Duffraisse et al investigates the role of a putative nuclear export signal in the activity of the hox gene Ubx and to a certain extent also in other hox genes. The authors hypothesise that the control of nuclear localization by nuclear export may present another layer of regulation for hox genes, as they are active as transcription factors in the nucleus but not in the cytoplasm. This is an interesting hypothesis, as several transcription factors are specifically regulated by nuclear import, but few if any are inactivated by specific nuclear export.

In summary, the authors make good points for their interesting hypothesis. I am however not convinced about the strength of some of the assays (nuclear localization in embryonic epidermis). The physiological relevance of the detected Ubx-Emb interaction remains doubtful to me. Importantly, the authors cannot clearly establish a correlation of nuclear export and loss of Ubx activity. More and better controls would be required for demonstration of specificity.

Comments for the author

To test their idea, the authors employed two in vivo assays. Ubx expression is restricted in embryos to the abdominal segments. Ubiquitous expression leads to a transformation of a thorax segment to abdominal fate which is easily detected by the pattern and morphology of ventral denticles in the cuticle. This transformation effect served as an assay for Ubx activity. For the second assay small clones in the larval fat body were induced, allowing a direct comparison of experimental and wild type situation in the same tissue. Given the large cell size in the larval fat body, the subcellular localization of proteins could be convincingly assessed. As a functional readout in the fat body, the authors detected a marker for autophagy, which depends on the down regulation of Ubx in late third instar larvae.

The authors tested the transformation activity and nuclear localization of six N- and C-terminal truncations in comparison to full length of Ubx in the embryonic epidermis. They found that all truncations lost the transformation activity, albeit the smallest construct DN282DC retained nuclear localization similar to full length. The authors conclude that a nuclear export signal, which would be deleted in the smallest construct but shielded in the full length protein would be responsible. The authors identified within the so-called HX region a motif with hydrophobic amino acid residues, which potentially would serve as a binding site for the most common nuclear export factor (Crm1/Embargo).

Mutation of this site leads to a nuclear localization of all trunctions, but only the construct with the smallest truncation showed a weak transformation activity (this transformation (Fig. 2B should be convincingly documented and quantified). The larger truncations are inactive despite the nuclear localization.

With a fluorescence complementation assay as well as direct binding (supplement) the authors show that Ubx and the nuclear export factor bound in vivo and can bind in vitro. The version with the mutated putative NES had reduced binding efficiency. The relevance of this binding remains unaddressed however, since the interaction of full length Ubx does not have a physiological consequence, as the bulk of Ubx remains in the nucleus and no increase in nuclear localization or activity of the mutated Ubx (mutated putative NES) was detected.

The relation of nuclear localization and Ubx activity is also tested in the larval fat body. Towards the late larval stage (wandering larvae) Ubx is down regulated, and as a consequence autophagy is activated. Clonal RNAi mediated depletion of Emb as well as the proteolysis factor culling leads to persistence of Ubx and suppression of autophagy. The authors conclude that depletion of nuclear export of Ubx by Emb would be responsible for the developmental delay in the clonal cells. In my view this is an overinterpretation, since Emb (as well as culling) are likely to affect many other factors beside Ubx. The effect is likely a delay in the developmental program. Given the developmental delay, Ubx is not transcriptionally down regulated and thus remains expressed and suppresses autophagy. The authors need to demonstrate specificity for Ubx, for example, by

showing that other proteins change during this stage (up-or down regulation) but not Ubx. The authors also need to show that persistence of Ubx proteins in emb RNAi clones is not based on persistence of Ubx RNA expression.

Lastly the authors test their constructs in the fat body assay. They compare nuclear expression and an autophagy marker for full length and a truncated Ubx with and without the putative NES. The authors claim that for both constructs mutations of the NES leads to a higher nuclear localization and stronger suppression of autophagy. What is missing here is the control of protein concentration. It is possible and visible in the images that the proteins with mutated NES are more stable and thus present in higher amounts. In this case it would be expected that a higher activity (suppression of autophagy) is observed. At least in the case of full length Ubx the statistical significance between the two versions, with and without NES should be calculated.

- The authors employ assays for the transformation activity by ubiquitous Ubx expression in an otherwise wild type background. The authors DO NOT test the function of their constructs, as no mutant complementation is conducted.

- The transformation assay is based on ubiquitous expression of the constructs. Nuclear localization and interaction with Emb is tested by Ubx-GAL4 driven expression. In my view this makes the comparison of the two assays and the interpretation of the data (functional relevance of nuclear localization) difficult, as the expression levels are likely not to be comparable. Higher expression levels of the constructs may change the nuclear localization, as this seems to be dose sensitive. Overexpression of Emb can lead to cytoplasmic localization of Ubx constructs (Fig. 2E). The nuclear localization and transformation activity should be investigated under similar conditions.

- It is not clear to me, why the co-expression of Emb in Fig2C+D did not lead to cytoplasmic localization where en-GAL induced expression led to nuclear export (Fig. 2E)

- the authors quantify the nuclear localization by the ratio of cytoplasmic and nuclear fluorescence. The nuclear region was assigned by DAPI fluorescence. This assay is fully convincing in case of the larval fat body, but not in case of the embryonic epidermis. In Figure 1C Ubx and Dapi staining are shown side-by-side. Even with full length Ubx the staining patterns do not match. The nuclear labelling (green for Ubx) and the nuclear labelling by DAPI are different. For full length Ubx (fist panel Fig. 1C) I observe seven nuclei with Ubx staining and at least nine with DAPI staining. The difference in pattern is also obvious in the overlay of the color channels. I would suggest, that the authors employ either a marker for the nuclear envelope as a nuclear marker or conduct the assays in the larval fat body.

Reviewer 2

Advance summary and potential significance to field

This is an interesting paper that uncovers a novel nuclear export signal (NES) in Hox proteins and demonstrates its relevance for the localization of the Hox protein Ubx in the Drosophila fat body regulating the onset of autophagy. By carrying out a dissection of Ubx using deletion constructs the authors identify a cryptic NES that is masked by N-terminal sequences in the full length protein. Mutation of residues predicted to be important for the NES does not affect the localization of full length Ubx, but rescues the nuclear localization of N terminal deletions. In a nice experiment, demonstrating the potential role of this NES, the authors show that overexpression of the Embargoed (Emb) exportin protein renders Ubx cytoplasmic, but nuclear accumulation is rescued by the NES mutation. In the fat body Emb RNAi identifies a role for nuclear export in the elimination of Ubx and release of the Ubx-dependent suppression of autophagy. Overall, this paper represents a significant advance in our understanding of the diversity of mechanisms regulating Hox function.

Comments for the author

Issues
1) I found the authors' presentation of the relationship between the novel NES and the previously described hexapeptide (HX) motif confusing. This is exemplified in the Abstract which is initially

clear that the novel NES and the HX are simply overlapping motifs, but then focuses on the HX motif: “HX….is involved in interaction with …Embargoed” and “HX-dependent nuclear export”. However, the mutated residues in the NES mutation in Ubx lie outside the HX. I think the authors should carefully consider the use of the term “motif” and bear in mind that the HX motif is, as the name indicates, a 6-amino acid motif. The author’s presentation causes problems in several places; e.g. in the title where “a versatile peptide motif” seems to imply that the HX and the NES are the same and on page 7 “To further confirm that the HX motif could work as an autonomous NES…” where the fused sequence extends outside the HX to encompass both the HX and the NES. While I agree that the overlap between the novel NES and the HX is interesting, and merits discussion, they are not the same sequence and it is misleading to present them as the same “peptide”.

2) The results are generally convincing however in the high-power views in Figs 1 and 2 I find it very difficult to correlate the DAPI labeled nuclei with the Ubx labeled nuclei. Is there a problem with the focus or the registration of these images?

3) Although there is discussion of the potential role of long disordered regions in controlling the NES activity, the authors should also discuss how the NES is proposed to have an effect in the fat body where it’s is clear from the difference between >VC-Ubx and >VC-Ubx{d235dC] that the N-terminal region is still masking the NES in this tissue.

Minor points
1) p3 line 13 “N-terminally fused” is confusing as it could be interpreted to mean that Ubx is N-term in the fusion construct.
2) p3 “these results confirm that the long N-terminal portion is required…”. I think it only shows a requirement for the N-terminal 130 AA.
3) The high power images in Fig 1-3 should have scale bars.
4) p6 “HX-mutated versions” requires an explanation of why these HX-mutated versions were used to study the NES.
5) p6 The authors should provide an explanation for why these HX-mutated versions “had no effect on the nuclear Hox-Emb interaction”.
6) Fig 3C and 3F panel headings; AntpGal4 should be enGal4.
7) “the HX motif is part of a functional NES in some but not all Hox proteins”. It’s not clear what the evidence for this statement is; there is no systematic testing for NES function and the NESs were not identified by conventional NES scanning.
8) p9 “ variable between different Drosophila Ubx isoforms”; it would be good to state which isoform was used for the deletion analysis and what isoforms are expressed in the embryo epidermis and in the larval fat body.
9) p10 “acquired independently in different Hox proteins”; as in point 7 above, it seems odd to argue that algorithms are not good at prediction and then to base a conclusion on their predictions.
10) Fig 1A. Is it right that regions in the N-terminal portion have higher conservation than the Homeodomain?
11) The boxes representing SLiMs appear to be offset from the SLiM peaks.
12) Figs1-3. What does “gamma”Ubx or GFP signify as a Figure label; if it stands for anti-Ubx then alpha-Ubx would be more conventional.
13) p12 Fig 2A legend NetNES plot; need to specify what the blue, green and red lines represent.
14) p13 Fig 2E legend “…expressed in posterior parasegments” ; not clear what this is trying to say as en is expressed in posterior compartments in all parasegments.

Reviewer 3

Advance summary and potential significance to field

Hox factors are major regulators of embryonic development. They specify cell fate and morphogenesis famously along the longitudinal axis of cnidarian and bilaterian animals. These factor recognise similar AT rich sequences in vitro, yet select and regulate diverse target gene in vivo, resulting in divergent morphology of the animal along the axis. Hox factors use TALE HD containing factors Pbc and Meis to carry out in vivo roles with whom they interact through YPWM motif or HX motif. However, as pointed out in the manuscript, Pbc and Meis interact with a large

majority of Hox proteins and therefore cannot account for full specificity of Hox regulatory repertoire.
Recently, it has been shown by the authors and some others that Hox factors are capable of interacting with other non-HD cofactors as well. However, the role of HX motif and other surrounding regions of HD in determining the interaction of Hox proteins with their cofactors and subsequent target gene selection is not fully understood. This manuscript is centered broadly around addressing this problem. In this work, the authors have identified a Nuclear Export Signal overlapping with the HX motif of one of the Drosophila Hox proteins Ubx. They subsequently characterize its contribution to Ubx nuclear localization and finally, its role in autophagy of larval fat body. They initially establish the role of this newly identified NES in determining the nuclear export of Ubx. They then go on to show that Ubx interacts with the nuclear transporter CRM1/emb through this HX motif and this interaction plays a role in nuclear localization of Ubx; eventually linking it to autophagy in larval fat body. They further extend the role of HX motif in nuclear export of two other Drosophila Hox factors, Scr and Deformed and also find that mutation of HX motif (NES) in human Hox proteins affects its nuclear localization as well. Using this study, authors have made an argument for the use of different molecular modules by Hox to carry out different versatile functions.

Overall, we find that the phenomenon described in this manuscript is novel and significant and that the manuscript is suitable for publication in JCS but in our opinion, this manuscript should go through major revision to facilitate understanding by a broader audience.

Comments for the author

The text, figures, and material and methods could have been better prepared. The Manuscript is very difficult to read and understand in its current form. We have three major concerns which are shown in bold in the suggestions below.

In fig1, it is not very clear why the A1 specification data (cuticles) is shown, it is not very informative and does not correlate well with the subsequent nuclear localization data of Ubx.

The cuticle data along with fig1A (which is not very well explained) should be moved to supplementary and the deletion schematic from figS1 should be in fig1 for readers to understand the subsequent experiment and analysis. It is very difficult to understand the extent of deletions made from the schematic given in Fig1. Even in fig2, the cuticle data is not very helpful and is diverting attention from some of the main conclusions.

One of the main conclusions of this figure is that N-terminal region is necessary for A1 specification. This is expected, considering many similar analyses with different Hox genes have been reported (Gibson and Gehring in 1990, being one of them; PMID: 1976044). Also, considering the data of dN235dC and dN130dC (which lack first 235 and 130 aa respectively and C-term regions), both of which are unable to specify A1 character, one can argue that 1-130 aa and not full N-term are important for A1 specification. One way will be to make just 1-130 aa deletion and see what is the cuticle looking like.

One of the conclusions of the authors is that region between 235-282 have an instructive role in nuclear localization, in that case they should delete 235-282 aa (leaving the HX motif intact) only and check the nuclear localization and A1 specification capacity of this deletion.

In fig2, the rescue of the cytoplasmic localization by deletions by mutation of NES is significant. Subsequently, the over expression of emb with Ubx which takes Ubx to cytoplasm is also convincing. However, the western blot of the pulldown in figS1C which shows the reduction in interaction of Emb with Ubx needs improvement.

One can argue that reduction in Emb-UbxNES interaction could be due to the fact that the amount of UbxNES in lane4 is less compared to lane3. It will help to have similar levels of Ubx and UbxNES in this blot (if not higher levels of UbxNES). A similar experiment should also be done for normal and HX mutant versions of Scr and Dfd to further substantiate the point.

In fig2D, it is not clear how BIFC quantitation of nuclear to cytoplasmic fraction is done in live embryos; it is not explained anywhere and reference to previous publications do not detail how this is to be done in live embryos. This should be detailed in Supplementary data if not in M&Ms. The same problem is there with Fig-3E and 3B as well.

Authors need to explain what they mean by and how are they are calculating repartition% and vesicules% in the graphs of fig4. It is not very clear.

Furthermore, EmbRNAi reduces autophagosome number and size, while cullinRNAi affects only the size and not the number. If Ubx is repressing autophagy in fatbody, then blocking Ubx degradation by cullinRNAi should have repressed both numbers and size of autophagosome, but that does not seem to be the case, why?

Minor corrections:

Explain the SLIMPred analysis in some details, fig1A needs to be explained.

It will helpful for the non-experts to appreciate the study if some explanation is provided on why Ubx was chosen for this analysis.

Comment on whether making an internal deletion of 130-235 and dC results in A1 specification.

Moreover, while the UbxNES and UbxdC cuticles are shown, it will also help to show UbxHX mutant cuticles as well for comparison.

fig1C, 2C and 2E different panels could be labelled separately as C?, C?? or c, d, e, f, etc. This will make it easier to refer to them and understand the text.

Since DAPI is the main criteria to assess the nuclear vs cytoplasmic localization, DAPI channel should be shown in fig2E and all subsequent figures.

How significance value was calculated, what are the error bars showing needs to be clarified somewhere.

Scale bar are shown what is their size?

In the inset for fig2D, cell boundaries are not very clear; it will help to clarify in methods what was chosen as the background and how the graphical quantitation was arrived at.

Representative BIFC image for UbxGal4> VC-UbxNES + VN-Emb could be made a little brighter.

In fig3C and 3F, authors say that they have used AntpGal4 but the pattern seems to be that of EnGal4. Please correct.

It is not very clear that why for Ubx, the three residues of NES are mutagenized leaving HX motif mostly intact while in Dfd and Scr residues lying within the HX was mutated, leaving predicted NES sequences mostly intact. Some explanation will be helpful.

In fig4 legend, author say that ?dotted staining corresponds to autophagosomes?, I thought the dotted line shows the FRT clone. Kindly clarify.

A final summary chart, may be in supplementary data showing the name of the deletions analysed, their nuclear/cytoplasmic localization, A1 specification capacity (if checked), if their NES mutation was made and what was the effect of this mutation on its localization (N/C) and its cuticle phenotype (if it was looked at).

Author have only checked the interaction of Ubx with emb, and they have shown presence of HX-NES motif and its role in Scr, Dfd and human HoxA5, in our opinion word ?several? can be misleading and should be replaced by ?multiple? in abstract.

?Here we show that the HX motif is involved in the interaction with the major CRM1/Embargoed exportin protein. This novel role was found in several Drosophila and human Hox proteins. We provide..?

Discussion sounds repetitive at places and the writing needs to be more precise. May be authors can try and save some words here and use them to better explain some of their results.

First revision

Author response to reviewers' comments

Reviewer-1
The first main concern of the Reviewer-1 was about the relevance/physiological consequence of the interaction assays in the embryo. We now use the embryo as a context to reveal the cytoplasmic localization of deleted forms of Ubx (since these forms are degraded in fat body cells) and to genetically provide evidence of the role of the unconventional NES in mediating the interaction with Emb/CRM1 in vivo (new Fig. 3 and Fig. S2). With this rational, we voluntary deleted the cuticle analysis part since it has no functional relevance with the role of the nuclear export in the embryonic epidermis.

The second main concern was about Cullin and Embargoed RNAi experiments in the fat body. We agree that these genetic contexts may affect the timing of developmental autophagy, leading indirectly to Ubx expression in feeding-delayed fat body cells. We would like to strengthen that the observation of Ubx in the cytoplasm of Cullin-RNAi cells still demonstrates that Ubx is actively degraded once exported, which was the purpose of the experiment. Inhibiting Emb could potentially inhibit the nuclear export of Ubx transcriptional repressor(s), a concern also raised by the Reviewer-3. To confirm that the effect was not due to a delay in developmental autophagy, we co-expressed a Ubx-deleted form that is normally actively exported and degraded (construct VC-UbxdN130dC), together with Emb-RNAi: in this genetic background, the presence of the construct in the nucleus demonstrates that removing Emb indeed inhibits the nuclear export independently of the developmental stage. Because this point is of major importance to demonstrate the physiological relevance of Hox nuclear export in the fat body, we included additional data about the role of CBP/p300: removing CBP/p300 induces cytoplasmic localization of endogenous Ubx or co-expressed VC-Ubx, but not of co-expressed NES-mutated VC-Ubx at the L3-F stage (new Fig. 4).
The third main concern of the Reviewer-1 was related to the precise quantification and comparison of the nuclear expression of Ubx constructs in fat body cells, in particular between wild type and NES mutated full-length forms. We did not systematically provide comparative statistics between the different constructs since we showed the proportion localized in the nucleus versus the cytoplasm in each case. The effect of the NES mutation is evident in the context of the deleted forms. We now mention in the text the statistical enrichment of the NES mutated form compared to the wild type in the context of full length Ubx.
Other points:

- Two concerns were related to the transformation activity under ectopic expression and the comparison with Ubx-Gal4 driven expression. Given that we do not show the cuticle and now use the embryonic staining to show that N-terminally deleted forms could be found in the cytoplasm when not degraded, these concerns do not apply in the revised version.
- One concern was about the co-expression of Emb and the cytoplasmic localization when using Ubx-Gal4 or En-Gal4 (new Fig. 3). The reviewer was confused by the fact that we showed in parallel Ubx-Gal4 with NES-mutated forms (previous panel C) and En-Gal4 with either wild type or the NES-mutated form together with Emb (previous panel E). In fact, the co-expression of Emb led to cytoplasmic localization of the VC-Ubx construct when using the two drivers. The forced cytoplasmic localization was lost upon the NES mutation either by doing BiFC (previous panel D) or by doing immunostaining (a BiFC-independent test, previous panel C). It is now more clearly shown in the new Fig. 3, which is fully dedicated to the NES-dependent interaction of Ubx with Emb in vitro and in vivo (panels A-C).
- The last concern was about the DAPI nuclear staining in the embryo. This concern does not apply anymore since the precise subcellular localization of Ubx constructs was performed in the

fat body cells, as suggested by the reviewer. This is now shown in the new Fig. 1 and Fig. 2. We would like to mention that the apparent discrepancy between DAPI and Ubx staining in the embryo is due by the fact that the Ubx-Gal4 driver is not expressed in all cells of the epidermis and also because the DAPI staining was realized by diffusion from the mounting medium: although very convenient, this way of staining is not always 100% efficient.

Reviewer-2
Major points:

- A main concern was about the confusion between the HX motif and the NES, which was interpreted as two different motifs by the reviewer. We tried to clarify this point by first changing the name “HX motif” by “Trp (W)-containing motif”, which is more neutral regarding the length. In any case, the fact that the W-containing motif is partially (in Ubx) or fully (in Scr, Dfd and HOXA5) included in the NES implies that it is involved in the nuclear export. This is the only important point that we tried to make as clear as possible: the fact that a conserved motif is part of a NES and could therefore participate to the control of Hox nuclear export in addition to its previous known PBC-recruiting functions. To better clarify this novel role of the W-containing motif, we added analyses with the classical/historical mutation (YPWM into YAAA in Ubx). This mutation affects only one core hydrophobic residue of the NES, and is still able to rescue the nuclear localization of the dN130dC deleted form, demonstrating the implication of the core residues of the W-containing motif in the NES activity (new Fig. 2).
- The Reviewer-2 raised the same point as the Reviewer-1 about the difficulty to correlate DAPI and Ubx embryonic staining in the enlargements. Please refer to our explanations for the Reviewer-1 regarding this particular this point. We have also changed the picture in each enlargement by tacking only one stack and not the full projection of confocal acquisitions for getting higher quality images more focused on the nuclei of interest.
- The Reviewer-2 proposes to discuss more about the masking role of the N-terminal region against the NES. Additional data (new Fig. 4 and Fig. 5) now better describe the role and the regulation of the N-terminal masking region during L3-F and L3-W stages.

Minor points: all minor points have been taken into consideration in the revised version.

Reviewer 3
Major concerns:

- The first major concern is about the region between 235-282 residues, which contains the unconventional NES. The Reviewer proposes to delete this region and analyze the consequence on A1 specification and nuclear localization. This deletion will in fact correspond to the IVa isoform of Ubx, which is the shortest Ubx isoform in Drosophila. This isoform has already been described as being correctly localized in the nucleus and was used to show the importance of the linker region in controlling the interaction mode with the PBC cofactor (Saadaoui and al., PNAS 2011 Feb8; 108(6): 2276-81). Since Ubx is constantly localized in the nucleus of embryonic epidermal cells, removing the major part of the NES with this deletion will not change the nuclear localization, and therefore not provide any useful information. The problem here is the lack of the physiological significance of the nuclear export in the embryonic epidermis, which diluted the central message and induced confusion. The revised version is now centered on the fat body for better clarity.
- The second main concern was related to the GST pull down experiments. In the gel used for the picture, there was indeed less produced NES-mutated Ubx, which was taken into account for quantification of the interaction with Emb. Nevertheless, we repeated two more times the experiment, together with the pull down of Scr (three independent experiments), allowing us to statistically quantify with more robustness the effect of the NES mutation on the interaction with Emb in vitro. Results are presented in the new Fig. 3 and Fig. S3. We focused on Ubx and Scr and repeated the GST pull down experiment several times instead of doing also Dfd with less repeated experiments, considering that the context with Scr and Dfd is highly similar (the HX motif is identical and fully included in the NES).
- More details have been provided for BiFC quantification with FIJI. We also refer to a chapter of a protocol book.
- There was a mistake in the quantification of vesicles’number in Cullin RNAi clones and we thank the reviewer for her/his observation. It has been corrected in the new Fig. 4.

Minor points: all minor points, except the final summary chart, have been taken into consideration in the revised version. Given the focus of the revised version on fat body cells and autophagy, we

preferred making a final figure illustrating the role and the control of the NES-dependent nuclear export in this tissue (new Fig. 5A).

Second decision letter

MS ID#: JOCES/2019/241943

MS TITLE: Role of a versatile peptide motif controlling Hox nuclear export and autophagy in the Drosophila fat body

AUTHORS: Marilyne Duffraisse, Rachel Paul, Julie Carnesecchi, Bruno Hudry, Agnes Banreti, Jonathan Reboulet, Leiore Ajuria, Ingrid Lohmann, and Samir Merabet
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, reviewers #1 and #3 consider that many of their major criticisms have not been addressed in your revised manuscript. Thus, further amendments to your work are still required. I also call your attention to the importance of doing a point by point rebuttal. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Unfortunately, the authors did not respond point-to-point to my criticism and suggestions. I had to sort out the various changes in the revised version my self and associate them to my suggestions. Unfortunately several of my concerns have remained unanswered and were not addressed. Along the lines explained in my initial evaluation, central conclusions are not convincingly supported by the data. In my view these conclusions represent unjustified overinterpretation.

Comments for the author

My initial comments start with >>

>>With a fluorescence complementation assay as well as direct binding (supplement) the authors show that Ubx
>>and the nuclear export factor bound in vivo and can bind in vitro. The version with the mutated putative NES
>>had reduced binding efficiency. The relevance of this binding remains unaddressed however, since the i>>nteraction of full length Ubx does not have a physiological consequence, as the bulk of Ubx remains in the
>>nucleus and no increase in nuclear localization or activity of the mutated Ubx (mutated putative NES) was
>>detected.
The authors did not respond to my concern in their rebuttal letter. The issue of the relevance of the binding remains unaddressed. The new Fig. 3 presents the interaction data between UBX and EMB, and the reporter assay showing the NES activity of the peptide sequence, but no data concerning the physiological relevance such a correlation of NES activity and UBX function.

>>The relation of nuclear localization and Ubx activity is also tested in the larval fat body. Towards the late larval
>>stage (wandering larvae) Ubx is down regulated, and as a consequence autophagy is activated. Clonal RNAi
>>mediated depletion of Emb as well as the proteolysis factor culling leads to persistence of Ubx and
>>suppression of autophagy. The authors conclude that depletion of nuclear export of Ubx by Emb would be
>>responsible for the developmental delay in the clonal cells. In my view this is an overinterpretation, since Emb
>>(as well as culling) are likely to affect many other factors beside Ubx. The effect is likely a delay in the
>>developmental program. Given the developmental delay, Ubx is not transcriptionally down regulated and thus
>>remains expressed and suppresses autophagy. The authors need to demonstrate specificity for Ubx, for
>>example, by showing that other proteins change during this stage (up-or down regulation) but not Ubx.
The
>>authors also need to show that persistence of Ubx proteins in emb RNAi clones is not based on persistence of
>>Ubx RNA expression.
The authors did not directly respond to my concern that developmental timing is changed in Embryos depleted clones. The authors added new data showing that CBP prevents nuclear export of UBX. These data are not related to the problem of developmental timing, however.
Also the authors did also not conduct the control of UBX transcripts. They do not even mention this requested experiment in their rebuttal letter.
Thus, it is likely in my view that the changes in autophagy is due to changes in the developmental timing but not to the nuclear export of UBX.

>>Lastly the authors test their constructs in the fat body assay. They compare nuclear expression and an
>>autophagy marker for full length and a truncated Ubx with and without the putative NES. The authors claim
>>that for both constructs mutations of the NES leads to a higher nuclear localization and stronger suppression
>>of autophagy. What is missing here is the control of protein concentration. It is possible and visible in the
>>images that the proteins with mutated NES are more stable and thus present in higher amounts. In this case it
>>would be expected that a higher activity (suppression of autophagy) is observed. At least in the case of full
>>length Ubx the statistical significance between the two versions, with and without NES should be calculated.

The authors do not directly respond to the issue of absolute protein levels. Even if the ratio of nuclear/cytoplasmic UBX is decreased, the absolute amount of nuclear UBX is higher in case of higher total UBX levels. Measuring nuclear - cytoplasmic ratios is only informative in cases of similar or at least comparable total proteins levels. The data as presented do not rule out this possibility.

>>The authors employ assays for the transformation activity by ubiquitous Ubx expression in an otherwise wild
>>type background. The authors DO NOT test the function of their constructs, as no mutant complementation is
>>conducted.
No response by authors

>>the authors quantify the nuclear localization by the ratio of cytoplasmic and nuclear fluorescence. The
>>nuclear region was assigned by DAPI fluorescence. This assay is fully convincing in case of the larval fat
>>body, but not in case of the embryonic epidermis. In Figure 1C Ubx and Dapi staining are shown side-by-
side.
>>Even with full length Ubx the staining patterns do not match. The nuclear labelling (green for Ubx) and the
>>nuclear labelling by DAPI are different. For full length Ubx (fist panel Fig. 1C) I observe seven nuclei with Ubx
>>staining and at least nine with DAPI staining. The difference in pattern is also obvious in the overlay of the
>>color channels. I would suggest, that the authors employ either a marker for the nuclear envelope as a
>>nuclear marker or conduct the assays in the larval fat body.
The explanation by authors is not convincing, however fig 1 has been removed in the revised version

Reviewer 2

Advance summary and potential significance to field

The authors have improved the manuscript and have generally satisfactorily addressed my comments.

Comments for the author

A few minor points remain:

1) Near end of first section of Results. The description of N-term deletions and location of NES is confusing. "Thus N-terminal deletions lead to cytoplasmic localization of Ubx, ...These observations suggest the presence of a nuclear export signal (NES) in the N-terminal part of Ubx" I understand what the authors are trying to say and that "N-terminal deletions" and "N-terminal part" refer to different things but it is confusing and could be expressed more clearly.

2) Fig 3B legend The authors have changed the text in response to my comment but it is still not correct. En is expressed in posterior compartments which are defined according to segments, and lie in the anterior part of parasegments. So "the posterior part of all parasegments" should be changed to "the anterior part of all parasegments"

3) Fig S1A Should the labels in the first panel be mCherry not GFP?

4) FigS1 In B there is the boxed sequence FYWPMA then in C we have FYPWMA.

Reviewer 3

Advance summary and potential significance to field

Summary of the advance made in this paper and its potential significance to the field.

Hox factors are major regulators of embryonic development. They specify cell fate and morphogenesis famously along the longitudinal axis of cnidarian and bilaterian animals. These factor recognize similar AT rich sequences in vitro, yet select and regulate diverse target gene in vivo, resulting in divergent morphology of the animal along the axis. Hox factors use TALE HD containing factors Exd/Pbc and Hth/Meis to carry out in vivo roles with whom they interact through YPWM motif or HX motif. However, as pointed out in the manuscript, Pbc and Meis interact with a large majority of Hox proteins and therefore cannot account for full specificity of Hox regulatory repertoire.
Recently, it has been shown by the authors and some others that Hox factors are capable of interacting with other non-HD cofactors as well. However, the role of HX motif and other surrounding regions of HD in determining the interaction of Hox proteins with their cofactors and subsequent target gene selection is not fully understood. This manuscript is centered broadly around addressing this problem. In this work, the authors have identified a Nuclear Export Signal overlapping with the HX motif of one of the Drosophila Hox proteins Ubx. They subsequently characterize its contribution to Ubx nuclear localization and finally, its role in autophagy of larval fat body.
They initially establish the role of this newly identified NES in determining the nuclear export of Ubx. They then go on to show that Ubx interacts with the nuclear transporter CRM1/emb through this HX motif and this interaction plays a role in nuclear localization of Ubx; eventually linking it to autophagy in larval fat body. They further extend the role of HX motif in nuclear export of two other drosophila Hox factors, Scr and Deformed and also find that mutation of HX motif (NES) in human Hox proteins affects its nuclear localization as well. Using this study, authors have made an argument for the use of different molecular modules by Hox to carry out different versatile functions.

Overall, we find that the phenomenon described in this manuscript is novel and significant and that the manuscript is suitable for publication in JCS but in our opinion, this manuscript should go through some minor revision to facilitate understanding by a broader audience.

Comments for the author

Role of a versatile peptide motif in controlling Hox nuclear export and autophagy in the Drosophila fat body Marilyne Duffraisse, Rachel Paul, Bruno Hudry, Julie Carnesecchi, Agnes Banreti, Jonathan Reboulet, Leiore Ajuria, Ingrid Lohmann and Samir Merabet.

Usually it is advisable to do a point by point rebuttal which has not been done by the author, therefore some of our concerns have been re-highlighted below, it will be helpful for readers if these concerns are addressed.

In general manuscript writing has greatly improved especially with removal of cuticle data. We are in favor of accepting the revised manuscript.
However, there are still some issues that need to be addressed.

When they start out with the results, their aim is to understand what determines Hox generic functions. However, they ultimately describe the use of NES for context specific interaction, this was little confusing. In the previous paper (Banreti 2014), they have shown Dfd, Ubx and AbdA expression in fat body. May be they should discuss whether they think that the identified NES in Dfd plays a role in autophagy repression. If yes, then the NES can indeed be believed to be a determinant of generic Hox function.

How are authors claiming that Ubx could interact with Emb in both cytoplasm and nucleus? Also how are they able to quantitate BIFC interaction cytoplasm and the fact that it is abolished in Ubx-NES, especially if no DAPI staining is shown.

Looking at Fig-3A, this is not very clear and obvious.

Authors can explain in some detail what they mean by and how are they are calculating repartition% and vesicules% in the graphs of fig4. It is not very clear. These details can be in supplementary data.

Minor points
In most figures, individual panels have still not been labelled, hence it becomes difficult to correlate text with figures. Different panels could be labelled separately as C', C'' C''' etc. This will make it easier to refer to them and understand the text and correlate it to the figures.
Scale bar are shown what is their size? Scale bars not shown in Fig3 and not mentioned in fig4 and supp fig.

Page-9, line-6 from top, authors are using emb RNAi for knockdown and this is referred to as emb mutant in subsequent text. This needs to be corrected it is a knockdown and should not be referred to as mutant data. The same is the case for Cullin RNAi data as well.

Addition of CBP knockdown data is a real value addition to manuscript. It will be helpful to have a line in main text saying that loss of CBP knockdown advances autophagy into L3-F stage. Currently this is not coming out explicitly in results though it is mentioned in discussion.

Material and Methods mentions about using the following RNAi lines: UAS-NemoRNAi, P(KK104885); UAS-PKA-C3RNAi, P(Trip.JF02723); UAS-NemoRNAi P(KK104885); UAS-CKIIRNAi,P(KK106845); UAS-CBPRNAi, P(KK105115)).
Other than CBPRNAi results for none of the other RNAi lines has been shown.
May be this needs to be corrected.

"syntax" need to be improved at places.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field

Unfortunately, the authors did not respond point-to-point to my criticism and suggestions. I had to sort out the various changes in the revised version my self and associate them to my suggestions. Unfortunately several of my concerns have remained unanswered and were not addressed. Along the lines explained in my initial evaluation, central conclusions are not convincingly supported by the data. In my view these conclusions represent unjustified overinterpretation.

We did not reply by a point-by-point rebuttal letter to the reviewer's concerns that were related to the physiological relevance of Hox-Emb interaction in the embryonic epidermis because this aspect was corrected in the fat body. The physiological relevance was also a concern raised by the reviewer 3, and we decided to completely reshape the paper by focusing on the fat body and adding convincing data showing the physiological relevance of Hox-Emb interaction for controlling autophagy. Assays in the embryonic epidermis were kept for molecular validation and justified by the genetic tools that could be used in this context and not in the fat body developmental context. We now provide a point-by-point response to the reviewer 1'comments.

Reviewer 1 Comments for the author
My initial comments start with <<

<<With a fluorescence complementation assay as well as direct binding (supplement) the authors show that
Ubx

<<and the nuclear export factor bound in vivo and can bind in vitro. The version with the mutated putative
NES
<<had reduced binding efficiency. The relevance of this binding remains unaddressed however, since the
i<<nteraction of full length Ubx does not have a physiological consequence, as the bulk of Ubx remains in the
<<nucleus and no increase in nuclear localization or activity of the mutated Ubx (mutated putative NES) was
<<detected.
The authors did not respond to my concern in their rebuttal letter. The issue of the relevance of the binding
remains unaddressed. The new Fig. 3 presents the interaction data between UBX and EMB, and the reporter
assay showing the NES activity of the peptide sequence, but no data concerning the physiological relevance
such a correlation of NES activity and UBX function.

We voluntary did not show any data related to the physiological relevance of Ubx-Emb in the embryonic epidermis because there is no such relevance in this developmental context (Hox proteins are located in the nucleus over embryonic developmental stages). Still this tissue presents several advantages for dissecting the interaction in a complementary approach to in vitro approaches: BiFC and genetic assays, even not in the appropriate tissue, still consolidated the molecular observation that the two proteins can interact in a NES-dependent manner. The goal of the Figure 3 is strictly limited to this aspect. The same rational applies to the analysis of NES peptides, although we showed their activity in the fat body in the revised version (new Fig. 3D"). This is why we voluntary deleted the functional consequences in the cuticle phenotypes since there is no Ubx export in the epidermis, to better focus on the functional consequences in the relevant tissue in the new Figure 4 (see below).
BiFC was performed in the embryonic epidermis and not the fat body because we have all the genetic tools to express Ubx at normal doses and to precisely quantify BiFC in vivo. We don't have such tools for the fat body (the UbxGal4 driver is not expressed in the fat body). Ectopic experiment with en-Gal4 could have been done in the fat body (since it relies on non-controlled expression levels), but the genetic evidence was in any case defintively demonstrated in the Figure 4 (see below).

<<The relation of nuclear localization and Ubx activity is also tested in the larval fat body. Towards the late
larval
<<stage (wandering larvae) Ubx is down regulated, and as a consequence autophagy is activated. Clonal
RNAi
<<mediated depletion of Emb as well as the proteolysis factor culling leads to persistence of Ubx and
<<suppression of autophagy. The authors conclude that depletion of nuclear export of Ubx by Emb would be
<<responsible for the developmental delay in the clonal cells. In my view this is an overinterpretation, since
Emb
<<(as well as culling) are likely to affect many other factors beside Ubx. The effect is likely a delay in the
<<developmental program. Given the developmental delay, Ubx is not transcriptionally down regulated and
thus
<<remains expressed and suppresses autophagy. The authors need to demonstrate specificity for Ubx, for
<<example, by showing that other proteins change during this stage (up-or down regulation) but not Ubx.
The

<<authors also need to show that persistence of Ubx proteins in emb RNAi clones is not based on persistence
of
<<Ubx RNA expression.

We specifically addressed this point in the revised version, which was clearly an important point, and which was also at the basis of our choice to add the data with CBP (see below). Following the rational of the reviewer, blocking the export will block/delay the developmental stage, and the fat body will remain at the L3-F stage, thus showing endogenous Ubx in the nucleus. The same rational applies for Cullin-RNAi.
Regarding Cullin-RNAi, and as previously mentioned in the Editor letter, the goal of this experiment was just to show that the nuclear export is tightly linked to degradation, which we could demonstrate by observing cytoplasmic Ubx (Fig. 4B). We could never see cytoplasmic Ubx at the L3-F or L3-W stage in wild type larvae.
We agree with the reviewer that blocking Emb could affect many other processes and, indirectly, affect nuclear localization of endogenous Ubx. That is why we co-expressed a form of Ubx that is normally constantly exported, degraded, and not able to repress autophagy (the VC-UbxdN235dC form: Fig. 1C). When co-expressed with Emb-RNAi, this form is located in the nucleus and able to repress autophagy (new Fig. 4A). This is strong evidence that the constant export of the VC-UbxdN235dC construct (due to the unmask of the NES) is fully dependent on Emb. Because we showed it with the UAS-driven construct, it also proves that this effect is not relying on any mechanism at the level of endogenous Ubx transcription. We believe this is a key result that definitively proves the importance of Emb-dependent export of Ubx for controlling autophagy, all shown in the tissue of physiological interest.
In conclusion, the argument of the reviewer on the regulation of Ubx RNA expression and the resulting indirect effect on autophagy would have been valid if we did not show the different effects by using transgenic constructs that behaved differently depending on the integrity of the NES.

The authors did not directly respond to my concern that developmental timing is changed in Embryos
depleted clones. The authors added new data showing that CBP prevents nuclear export of UBX. These data
are not related to the problem of developmental timing, however.
Also the authors did also not conduct the control of UBX transcripts. They do not even mention this requested experiment in their rebuttal letter.
Thus, it is likely in my view that the changes in autophagy is due to changes in the developmental timing but
not to the nuclear export of UBX.

We agree that the developmental time might be affected, therefore affecting the expression of endogenous Ubx transcripts. This is why we confirmed the observation of endogenous Ubx by conducting UAS-driven experiments: this genetic system avoids being dependent of any indirect effect on mRNA expression, as previously mentioned for EmbRNAi. In this context, co-expressing UAS-VC-Ubx with CBP-RNAi led to strong cytoplasmic localization and autophagy, despite the strong expression of Ubx (new Fig. 4C). We should say here that the simple expression of Ubx at the L3-W stage is sufficient to block autophagy, which clearly demonstrates that Ubx can still localized in the nucleus and repress autophagy independently of the developmental stage when ectopically/constitutively expressed. Therefore the effect observed with CBP-RNAi is specific. This effect is lost as soon as we used the NES-Ubx mutated form, demonstrating that the export of Ubx observed in the absence of CBP was fully dependent of the NES motif (new Fig. 4C). This result definitively proves that the interaction between Ubx and Emb occurs through the NES in the physiological tissue. As mentioned previously, using UAS-driven constructs makes the analysis and interpretation completely independent on the regulation of endogenous Ubx transcripts. We should have made this point clear in the previous rebuttal letter.

<<Lastly the authors test their constructs in the fat body assay. They compare nuclear expression and an
<<autophagy marker for full length and a truncated Ubx with and without the putative NES. The authors claim

<<that for both constructs mutations of the NES leads to a higher nuclear localization and stronger suppression
<<of autophagy. What is missing here is the control of protein concentration. It is possible and visible in the
<<images that the proteins with mutated NES are more stable and thus present in higher amounts. In this
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<<would be expected that a higher activity (suppression of autophagy) is observed. At least in the case of full
<<length Ubx the statistical significance between the two versions, with and without NES should be calculated.
The authors do not directly respond to the issue of absolute protein levels. Even if the ratio of nuclear/cytoplasmic UBX is decreased, the absolute amount of nuclear UBX is higher in case of higher total
UBX levels. Measuring nuclear - cytoplasmic ratios is only informative in cases of similar or at least comparable total proteins levels. The data as presented do not rule out this possibility.

We have provided the statistical significance between full length wild type and NES mutated Ubx constructs in the legend of the new Figure 2B-B' of the revised version: "Compared to wild type Ubx, the NES mutation induces statistically higher levels of nuclear localization and autophagy repression in the context of the full-length protein (p-value of 2,15E-10 and 4,58E-13, respectively) ".
The fact that the NES mutation increases nuclear Ubx, therefore decreases the amount of degraded cytoplasmic Ubx, makes sense with an indirect role in the stability of the protein, as shown with the Cullin-RNAi experiment. It is therefore difficult to specifically address the point raised by the reviewer: since the nuclear export is tightly linked to degradation we cannot compare the absolute amount of nuclear Ubx with the different constructs. The point here was to show that we could induce anticipated nuclear export when relieving the NES mask (by making dN130 and dN235 deletions), and that we could rescue this anticipated export by mutating the NES. Whether the NES could also stabilize the protein within the nucleus is another issue that is beyond the scope of this work.

<<The authors employ assays for the transformation activity by ubiquitous Ubx expression in an otherwise
wild
<<type background. The authors DO NOT test the function of their constructs, as no mutant complementation is
<<conducted.
No response by authors

We did not respond to this point because it is referencing to the cuticle assays, which are not functionally relevant with the Ubx export. We followed his/her recommendation (as mentioned in the Editor letter) to show assays only in the relevant fat body tissue. Unfortunately, there is no Ubx mutant background that could be used for complementation assays in the fat body (we need to abolish all redundant Hox genes involved in autophagy repression for this type of assay, which is genetically complicated and almost impossible unless we could express back Ubx and the derived constructs at physiological levels in the fat body). Ubx complementation assays could be performed in other developmental contexts, like for example the haltere rescue phenotype, but there is no export in this tissue, raising again the issue of the physiological relevance for this type of rescue assay.

<<the authors quantify the nuclear localization by the ratio of cytoplasmic and nuclear fluorescence. The
<<nuclear region was assigned by DAPI fluorescence. This assay is fully convincing in case of the larval fat
<<body, but not in case of the embryonic epidermis. In Figure 1C Ubx and Dapi staining are shown side-by-side.
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<<color channels. I would suggest, that the authors employ either a marker for the nuclear envelope as a
<<nuclear marker or conduct the assays in the larval fat body.
The explanation by authors is not convincing, however fig 1 has been removed in the revised version

We have justified why not using anymore the embryonic epidermis for this purpose (since there is no physiological role of the nuclear export in this context). The concern does not apply anymore since the precise subcellular localization of Ubx constructs was performed in the fat body cells, as suggested by the reviewer. This is now shown in the new Fig. 1 and Fig. 2. As the reviewer said, it is now fully convincing in the larval fat body.
Regarding our explanation for the discrepancy with DAPI staining in the previous Editor letter, the fact that Ubx-Gal4 is not ubiquitous in all the epidermis nuclei is simply true observation that has been reported in a number of other published work. The fact that DAPI from the mounting medium is not fully diffusing within all nuclei is also a pure practical observation, but we should have taken care of it during the confocal acquisitions.

Reviewer 2 Advance summary and potential significance to field
The authors have improved the manuscript and have generally satisfactorily addressed my comments.

Changes made in the text are highlighted in red in the point-by-point response to the reviewers 2 and 3.

Reviewer 2 Comments for the author
A few minor points remain:

1) Near end of first section of Results. The description of N-term deletions and location of NES is confusing. "Thus N-terminal deletions lead to cytoplasmic localization of Ubx, ...These observations suggest the presence of a nuclear export signal (NES) in the N-terminal part of Ubx" I understand what the authors are trying to say and that "N-terminal deletions" and "N-terminal part" refer to different things but it is confusing and could be expressed more clearly.

We agree with the reviewer that it could be confusing, and simplify the corresponding sentence: “These observations suggest the presence of a nuclear export signal (NES) that is active in the context of N-terminally deleted Ubx proteins.”

2) Fig 3B legend The authors have changed the text in response to my comment but it is still not correct. En is expressed in posterior compartments which are defined according to segments, and lie in the anterior part of parasegments. So "the posterior part of all parasegments" should be changed to "the anterior part of all parasegments"

We thank the reviewer for this mistake! We changed the figure legend accordingly.

3) Fig S1AShould the labels in the first panel be mCherry not GFP?

We thank the reviewer for this second mistake. The first panel in fact corresponds to the Ubx staining , as mentioned in the legend. We corrected the mistake in the Fig. S1.

4) FigS1 In B there is the boxed sequence FYWPMA then in C we have FYPWMA.

Indeed one more spelling mistake that we corrected in the Fig. S1.

Reviewer 3 Advance summary and potential significance to field
Summary of the advance made in this paper and its potential significance to the field.

Hox factors are major regulators of embryonic development. They specify cell fate and morphogenesis famously along the longitudinal axis of cnidarian and bilaterian animals. These factor recognize similar AT rich sequences in vitro, yet select and regulate diverse target gene in vivo, resulting in divergent morphology of the animal along the axis. Hox factors use TALE HD containing factors Exd/Pbc and Hth/Meis to carry out in vivo roles with whom they interact through YPWM motif or HX motif. However, as pointed out in the manuscript, Pbc and Meis interact with a large majority of Hox proteins and therefore cannot account for full specificity of Hox regulatory repertoire. Recently, it has been shown by the authors and some others that Hox factors are capable of interacting with other non-HD cofactors as well. However, the role of HX motif and other surrounding regions of HD in determining the interaction of Hox proteins with their cofactors and subsequent target gene selection is not fully understood. This manuscript is centered broadly around addressing this problem. In this work, the authors have identified a Nuclear Export Signal overlapping with the HX motif of one of the Drosophila Hox proteins Ubx. They subsequently characterize its contribution to Ubx nuclear localization and finally, its role in autophagy of larval fat body. They initially establish the role of this newly identified NES in determining the nuclear export of Ubx. They then go on to show that Ubx interacts with the nuclear transporter CRM1/emb through this HX motif and this interaction plays a role in nuclear localization of Ubx; eventually linking it to autophagy in larval fat body. They further extend the role of HX motif in nuclear export of two other drosophila Hox factors, Scr and Deformed and also find that mutation of HX motif (NES) in human Hox proteins affects its nuclear localization as well. Using this study, authors have made an argument for the use of different molecular modules by Hox to carry out different versatile functions.

Overall, we find that the phenomenon described in this manuscript is novel and significant and that the manuscript is suitable for publication in JCS, but in our opinion, this manuscript should go through some minor revision to facilitate understanding by a broader audience.

Reviewer 3 Comments for the author
Role of a versatile peptide motif in controlling Hox nuclear export and autophagy in the Drosophila fat body Marilyne Duffraisse, Rachel Paul, Bruno Hudry, Julie Carnesecchi, Agnes Banreti, Jonathan Reboulet, Leiore Ajuria, Ingrid Lohmann and Samir Merabet.

Usually it is advisable to do a point by point rebuttal which has not been done by the author, therefore some of our concerns have been re-highlighted below, it will be helpful for readers if these concerns are addressed.

In general manuscript writing has greatly improved especially with removal of cuticle data. We are in favor of accepting the revised manuscript. However, there are still some issues that need to be addressed.

When they start out with the results, their aim is to understand what determines Hox generic functions. However, they ultimately describe the use of NES for context specific interaction, this was little confusing. In the previous paper (Banreti 2014), they have shown Dfd, Ubx and AbdA expression in fat body. May be they should discuss whether they think that the identified NES in Dfd plays a role in autophagy repression. If yes, then the NES can indeed be believed to be a determinant of generic Hox function.

We thank the reviewer for this interesting point that we should have discussed a bit more. We completed the discussion section with a small paragraph regarding the link between the NES-activity of the W-containing region and the generic function of Hox proteins in the fat body: “The role of the W-containing motif as part of a docking platform for the Emb/CRM1 exportin protein enlarges the repertoire of its putative binding partners (Fig. 5B). This role was described for Ubx and Dfd, but no NES-like signature could be identified in the W-containing region of AbdA or AbdB, which are also autophagy repressors during the L3-F stage (Banreti et al., 2014). This suggests that the control of Hox nuclear export in the fat body could rely on NES that are not systematically located in the W-containing region, highlighting that the control of a generic Hox function could rely on different peptide motifs.”

How are authors claiming that Ubx could interact with Emb in both cytoplasm and nucleus? Also how are they able to quantitate BIFC interaction cytoplasm and the fact that it is abolished in Ubx-NES, especially if no DAPI staining is shown.
Looking at Fig-3A, this is not very clear and obvious.

The different BiFC profiles between VC-Ubx or VC-UbxNES and VN-Emb are evident on confocal acquisitions, but we agree more difficult to appreciate in the Figure 3A, due to the size of the panels. BiFC between VC-Ubx and VN-Emb is stronger in the nucleus, which allows recognizing the nucleus, and the signal also diffuses in the cytoplasm. The cytoplasmic diffusion is lost upon the NES mutation. Cytoplasmic BiFC was evident with the co-expression of mCherry, which stains both the nucleus and cytoplasm in expressing cells. It was voluntary not shown in the Figure 3A for simplification. Given that BiFC has to be quantified in live condition, we could not use DAPI. We used both the nuclear BiFC and the mCherry signal to make calques on Fiji (with the same principle as described for quantification of autophagy vesicles: see below) and specifically quantify BiFC either in the nucleus or in the cytoplasm for each condition. The quantification could not be based on a nuclear marker, since the co-expression of Emb systematically leads to the cytoplasmic localization of all tested red fluorescent reporters. We provide an additional supplementary Figure to better highlight the fluorescent profiles and describe the process of quantification with Fiji (new Fig. S4).

Authors can explain in some detail what they mean by and how are they are calculating repartition% and vesicules% in the graphs of fig4. It is not very clear. These details can be in supplementary data.

We described the full protocol used with Fiji to quantify the surface and number of autophagy vesicles. This information is now included in the paragraph “Flip-out expression of UAS constructs and mitotic clones and quantitation of protein localization and autophagy” of Materials and Methods section (highlighted in red in the tracked changes version of the ms).

Minor points
In most figures, individual panels have still not been labelled, hence it becomes difficult to correlate text with figures. Different panels could be labelled separately as C’, C’’ C’’’ etc. This will make it easier to refer to them and understand the text and correlate it to the figures.
Scale bar are shown what is their size? Scale bars not shown in Fig3 and not mentioned in fig4 and supp fig.

We had the rational of regrouping all images related to the same question, writing the different genotypes and staining around the pictures to easily follow each result (readers have to read the genotype, larval stage and type of staining for each figure panel in any case). This mode of presentation has several advantages: (i) it focuses on the same question in the same panel, (ii) it allows not saturating the pictures with writing inside, (iii) it allows not repeating the same writing in all images, (iv) it allows saving more space for each illustrative picture. This mode of presentation was also used in the previous publication (Banreti et al., Dev. Cell; 2014), allowing being homogenous between the two complementary studies. For all these reasons we prefer not changing the mode of presentation.
All missing scale bars have been added in the figures and mentioned in the figures’ legends.

Page-9, line-6 from top, authors are using emb RNAi for knockdown and this is referred to as emb mutant in subsequent text. This needs to be corrected, it is a knockdown and should not be referred to as mutant data. The same is the case for Cullin RNAi data as well.

We agree and corrected with the term “depleted”.

Addition of CBP knockdown data is a real value addition to manuscript. It will be helpful to have a line in main text saying that loss of CBP knockdown advances autophagy into L3-F stage. Currently this is not coming out explicitly in results though it is mentioned in discussion.

We mentioned the rational of the screen for finding post-translational modulators involved in autophagy regulation, but now formulated more explicitly the effect of CBP-RNAi:

The rational was that the inhibition of post-translational modifications involved in the NES-masking activity could lead to anticipated nuclear export of Hox proteins, thereby inducing anticipated autophagy at the L3-F stage. We observed that Ubx staining was affected in the nucleus, but also visible in the cytoplasm of CBP/p300-RNAi expressing cells at the L3-F stage (Fig. 4C). Atg8-mCherry revealed the formation of autophagy vesicles in the same CBP/p300-RNAi expressing cells (Fig. 4C), suggesting that anticipated autophagy could result from anticipated Hox nuclear export.

Material and Methods mentions about using the following RNAi lines: UAS-NemoRNAi, P(KK104885); UAS-PKA-C3RNAi, P(Trip.JF02723); UAS-NemoRNAi, P(KK104885); UAS-CKIIRNAi,P(KK106845); UAS-CBPRNAi, P(KK105115)).
Other than CBPRNAi results for none of the other RNAi lines has been shown.
May be this needs to be corrected.

We show illustrative pictures of the non-effect of PKA-C3-RNAi, CKII-RNAi and Nemo-RNAi on Ubx localization and autophagy in a new Fig. S5.

“syntax" need to be improved at places.

Syntax has been verified and changed when necessary.

Third decision letter

MS ID#: JOCES/2019/241943

MS TITLE: Role of a versatile peptide motif controlling Hox nuclear export and autophagy in the Drosophila fat body

AUTHORS: Marilyne Duffraisse, Rachel Paul, Julie Carnesecchi, Bruno Hudry, Agnes Banreti, Jonathan Reboulet, Leiore Ajuria, Ingrid Lohmann, and Samir Merabet
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers recognize that most of their initial criticisms have been addressed in your revised manuscript. However, they still raised issues that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 2

Advance summary and potential significance to field

See previous reviews

Comments for the author

The authors have satisfactorily addressed my comments....except could they double check the change to FigS1A. They appear to have made an appropriate change to the labels above the figure but the embryo panel still says GFP whereas I believe it should be "Ubx".

Reviewer 3

Advance summary and potential significance to field

The authors have satisfactorily addressed all of the concerns raised in the previous version of the manuscript, manuscript in our opinion is suitable for being accepted.

Comments for the author

Only suggestion being that authors should avoid referring to RNAi mediated knockdown as mutants (Pg 9 and Pg 11).

"...d the VC-UbxdN235dC construct in Emb mutant cells. In this context, the VCUbxdN235dC construct was retained in the nucleus of Emb mutant cells and able to repress autophagy (Fig. 4A)."

"...In addition, the induction of autophagy despite the constitutive expression of VC-Ubx in CBP/p300 mutant cells".

Third revision

Author response to reviewers' comments

We have amended the manuscript ("mutant" has systematically been replaced by "depleted" when referring to RNAi genotypes) and the Fig.S1A (it was indeed Ubx and not GFP in the first panel) according to the Reviewers' suggestions. We thank the reviewers for their careful reading and helpful suggestions.

Fourth decision letter

MS ID#: JOCES/2019/241943

MS TITLE: Role of a versatile peptide motif controlling Hox nuclear export and autophagy in the Drosophila fat body

AUTHORS: Marilyne Duffraisse, Rachel Paul, Julie Carnesecchi, Bruno Hudry, Agnes Banreti, Jonathan Reboulet, Leiore Ajuria, Ingrid Lohmann, and Samir Merabet
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.