The atypical histone variant H3.15 promotes callus formation in *Arabidopsis thaliana*

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MS TITLE: A novel histone 3 variant H3.15 controls plant regeneration in Arabidopsis thaliana

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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and 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. 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

There is substantial interest in understanding chromatin-based mechanisms that contribute to the regenerative potential of plants. The authors have found that expression of a histone variant of histone H3 that lacks lysine 27, H3.15, is induced in response to decapitation (wounding seems like

a bit of an understatement here) of an Arabidopsis seedling. They have also demonstrated that overexpression of H3.15 promotes callus formation. In light of these and other findings, the authors propose that this histone variant "controls plant regeneration in Arabidopsis thaliana" (taken from title). Although the presented data are intriguing, the functional characterization of H3.15 primarily relies on overexpression (and thus quite likely gain-of-function) data. Critically, no loss-of-function data are presented demonstrating that expression of H3.15 is necessary for (or normally contributes to) callus formation. Further, the authors observe that "restoring" lysine 27 to H3.15 fails to ameliorate the excessive callus phenotype in overexpression lines which is strongly in line with a gain-of-function phenotype resulting from overexpression of an altered histone that is not reflective of the lysine 27-dependent mechanism that is favored by authors. In light of these and other concerns (described below), the manuscript needs either a substantial amount of additional data or a substantial reduction of interpreted significance of findings prior to publication.

Comments for the author

If the authors are going to make any claims regarding the "role" of H3.15, they must include phenotypic characterization of a loss-of-function mutation (preferably a null) to substantiate the claim with regards to a wild-type context. Overexpression data are extremely suspect, particularly when accompanied by data demonstrating that reversion of the amino acid of interest does not suppress the phenotype of interest.
Another reason the authors need a loss-of-function mutant is that the presented data from the H3.15 reporter lines are extremely suspect. qRT-PCR data (Figure 1a) reveal that the endogenous transcript level of H3.15 is increased ~2-fold at 24h and ~4-fold at 48h relative to 0h. Yet the H3.15 reporter lines indicate no expression at 0h and thus a much greater fold-change at 24h and 48h (Figure 1c). The authors have defined the promoter region as the "1952bp genomic fragment upstream of the HTR15 coding region" (line 282), but have no evidence that this region is sufficient to replicate the normal expression pattern of HTR15 in the absence of a rescue data (there are numerous examples of plant genes in which DNA sequence embedded in the first intron or the 3' UTR are required for proper expression). At the very least, in situ data are needed to substantiate the data from the reporter lines.

As noted above, the observation that reversion of the conserved lysine residue has no effect on the overexpression callus enhancement phenotype (Figure 5) strongly detracts from the proposed model. In line with this, it is striking that the authors do not address whether or not H3.15 is conserved in plants. Absence of conservation would strongly argue that the observed overexpression phenotype is indeed gain-of-function and/or that the authors are not characterizing a mechanism of plant regeneration that is generally representative of the plant kingdom (a characteristic which is used to motivate this manuscript).

Additional comments:

Line 169: CAF1 does not exist to "prevent" callus formation. This is an extremely emergent phenotype resulting from loss of a key factor in chromatin assembly.

Line 201: pHTR15::HTR15 should likely read 35S::HTR15 based on annotation in Figure 6.

Line 310: Unclear as written. Is each data point a single biological replicate with 3 technical replicates or three biological replicates with 2 technical replicates? The former would be extremely suspect, the latter would be acceptable.

Figure 4B: What do dotted lines indicate?

Figure 3D (and related): please clarify how root length determined – is this of primary explant? Please describe in some detail how measure area of callus in methods.

Figure 4B: Non-callus control is needed for this comparative analysis.

Figure 6: These data are challenging to interpret in the absence of comparative controls. One has the sense that HTR15 is incorporated everywhere based on the use of TUB2 as comparative control in 6B and 6C.
Inclusion of H3K27me3 ChIP data for these loci would be very revealing as yet another comparative control.
Similarly, inclusion of H3K27me3 ChIP data for WT plants is needed for 6E to enable the reader to draw meaningfully inferences regarding the impact of overexpression of H3.15 on enrichment of H3K27me3.

Figure S1: Please clarify if panels B-E are all from 4d seedlings.

Figure S4C: What is expression relative to (y-axis)?

Figure S7C: What are criteria for scoring "callus" at 2d?

Figure S6 (and accompanying analysis): There is no obvious difference in expression of reporter genes between wild-type and transformed roots. Is the implication that the authors cannot replicate the findings of the 2010 paper? If they cannot, the implications of this observation should be addressed. Otherwise, if H3.15-enabled callus does follow a different pathway, this observation would once again strongly argue that this is a gain-of-function phenotype.

Reviewer 2

Advance summary and potential significance to field

This paper looks at the role of the histone variant H3.15 during regeneration in Arabidopsis and suggests that H3.15 promotes reprogramming and regeneration by reducing the repressive mark H3K27me3 (unlike H3.1, HTR15 lacks a lysine at residue 27 so can not carry the H3K27me3 modification) and so facilitating the activation of key regulators such as transcription factors. There is convincing data to show that HTR15 reporters are strongly induced by wounding or growth on callus inducing media (CIM), and that induction occurs in pericycle cells in roots, which are known to be important for regeneration on CIM. This is correlative evidence for a role of HTR15 in regeneration, the functional evidence mainly rests on over expression experiments using 35S:HTR15 and 35S:HTR15-KA (a modified version where the region around residue 27 is swapped with that of canonical H3.1, potentially allowing HTR15 to be methylated at K27), the former but not the latter giving rise to larger callus after wounding or growth on CIM.

I found it an interesting paper in an area of intense research interest and the central hypothesis (that HTR15 induction leads to HTR15 incorporation into chromatin and loss of H3K27me3 mediated repression of key genes promoting regeneration) is original and appealing. The data presented is consistent with this. However I did not find the data compelling, mainly because it relies very heavily on gain of function approaches and the characterization of the materials is limited. In its present form I don't think the paper is acceptable without addressing some of the issues below.

Comments for the author

Main points
1. A very obvious omission in the paper is the use of loss of function mutants or knock down lines to test the hypothesis that HTR15 is important for regeneration. There is no mention of this anywhere in the m/s. Redundancy does not seem to be an issue as HTR15 is unique amongst the histone variants, based on the phylogeny presented and also in terms of its induction by wounding. Testing whether reducing HTR activity by loss of function mutation (or conceivably artificial microRNA etc) can interfere with regeneration would add considerably.

2. Another striking omission is the lack of any mention that HTR15 also lacks a lysine at residue 4, so the H3K4me2 and H3K4me3 marks that are associated with gene activity are also likely to be reduced by incorporation of HTR15, instead the focus is very specifically on H3K27me3.

So it is not clear that incorporation of HTR15 would necessarily be activating and there is no attempt to look at effects on H3K4me3.

3. The evidence that HTR15 promotes regeneration is that 35S:HTR15 over-expression lines have large callus on wounded or CIM cultured tissue, and increased expression of some important transcription factors regulating regeneration. In all cases the assays seem to be performed a long time after induction (14 and 28 days for the callus assays, 40 days for the analysis in Fig S5, 8 days for the gene expression analysis). This seems late, so that the differences could be very downstream (for example, increase in cell division) rather than necessarily a greater ability to reprogram somatic cells. It would add to show whether the induction of genes like WOX11 is more rapid and more extensive using reporter lines.

4. There is very little description of the phenotype of the over expression lines. For example, western blot analysis of total histone extracts suggests that 35S:HTR15 lines have a very marked decrease in H3K27me3 levels in tissue grown on callus. Given that a large fraction of the genome is H3K27me3 decorated (at least in seedlings, it could be different in callus but probably not since the modification is readily detectable on histone blots) this would suggest that HTR15 must be replacing H3.1 at very many genes, not just at genes specifically involved in regeneration. Similarly, ChIP results using 35S::HTR15-FLAG retrieve chromatin at TUB2 at 2% of input, which might suggest that HTR15 may be widely incorporated in the genome at least in these lines. If this is the case one might expect 35S:HTR15 plants to show other phenotypes, but no description is provided of what these plants look like.

5. Another problem with the over expression lines is that HTR15 is now present at high levels in many cell types where it is not normally induced. So even if regeneration is facilitated it is not clear that this necessarily reflects the wild type function (in which the induction seems more specific to pericycle).

Minor points

There is no expression analysis for example to show that the transgenic lines giving a regeneration phenotype do have increased HTR15 levels.

The 35S:HTR15-FLAG line used for ChIP is very little characterised. Some kind of complementation analysis or even western blot to show expression of the fusion protein is usually included. In the ChIP experiments (Fig 6 B, C, F) there is an important control missing, i.e. using a non transgenic line for comparison in order to give confidence that the antibody is specifically pulling down HTR15-FLAG chromatin.

Figure 2B 48 hour samples – what do the white dotted lines indicate?

Figure 4C shows relative expression but does not indicate relative to what. Uninduced samples?

Figure 3B and D – why is one graph shown as a box plot and whiskers, the other as a bar chart?

Figure S7 – in part A it would be helpful to describe the stains, i.e. is the EdU Blue or pink, the latter presumably but helpful to point out and describe what the blue stain is. The text states that A and B support DNA replication at 24 hours, but I could not see induction of CYCB1;1 GUS until 72 hours? In part C it is claimed that fas2-4 has a different effect on callus induction to HTR15 OE, but the assay (callus formation rate %) is a different one to what was used for the HTR15 lines, so it is not clear the comparison is valid.

Figure S6 WOX5 and SCR reporter expression. It would be helpful to indicate which is the wounded (the ends?) and which the unwounded (the middle?) tissue. It was very unclear what this figure adds really, it does not seem to show any difference between wild type and HTR OE line and the differences between wounded and unwounded tissue don't add much since not followed up.

Reviewer 3

Advance summary and potential significance to field

A deep characterisation of the role of HISTONE THREE RELATED 15 (H3.15) during early wounding response and CIM-induced callus formation, in Arabidopsis hypocotyl and root tissues.
In summary, the manuscript offers novel data about H3.15 expression profile, with involvement of auxin in its expression regulation, the phenotypic effects of its ectopic expression, its roles on the regulation of H3K27 methylation, specifically for and on the expression of WOX5, WOX11, LBD16, LBD18, LBD29, and ERF115 possibly via direct H3K27 methylation.
These results are significant and of general interest for advancing our understanding of wound response and auxin-induced callus formation during in vitro, CIM-induced, regeneration.
Particularly interesting and relevant for a broad audience of developmental biologists is the clear link with epigenetic regulation.

Comments for the author

I believe this manuscript is suitable for publication, but I suggest minor revisions (below).

In general, the manuscript reads quite dense and the sequence of experimental results could be paced with more explicit rationales, but this is left to the personal styles of the authors. I would simply suggest the authors to see if there is room for smoother transitions from one experiment to another.

More specific comments for minor revisions:

- Lines 105-107 and Fig1.
Isn't Fig1 about early response to wounding, without CIM? If so, I don't see why mentioning callus at all. Is it visible in Fig1? Also, in Fig 1 it should more clear what is the difference between panels C, D, E. Finally, it would help to know more about the "wounding assay": where (meristem, elongation zone, etc) and how is the wounding generated?

- Lines 155-158 and Fig S6 It is unclear in S6 that the fate-specific expressions of WOX5 and SCR are different in 35S::HTR15 vs wt.
Also, even if this were true, why excluding the possibility that only those cells expressing WOX5 and SCR will participate to callus formation, either in WT or in 35S::HTR15?
Overall, the argument about a separate developmental pathway seems weak and unconvincing.

First revision

Author response to reviewers' comments

Dear Reviewers, we also uploaded a formatted PDF version of below responses as Supplementary Information in revision uploads.

Response to Reviews:

Reviewer 1 Advance summary and potential significance to field
There is substantial interest in understanding chromatin-based mechanisms that contribute to the regenerative potential of plants. The authors have found that expression of a histone variant of histone H3 that lacks lysine 27, H3.15, is induced in response to decapitation (wounding seems like a bit of an understatement here) of an Arabidopsis seedling. They have also demonstrated that overexpression of H3.15 promotes callus formation. In light of these and other findings, the authors propose that this histone variant “controls plant regeneration in Arabidopsis thaliana” (taken from title). Although the presented data are intriguing, the functional characterization of H3.15 primarily relies on overexpression (and thus quite likely gain-of-function) data. Critically, no loss-

of-function data are presented demonstrating that expression of H3.15 is necessary for (or normally contributes to) callus formation. Further, the authors observe that “restoring” lysine 27 to H3.15 fails to ameliorate the excessive callus phenotype in overexpression lines, which is strongly in line with a gain-of-function phenotype resulting from overexpression of an altered histone that is not reflective of the lysine 27-dependent mechanism that is favored by authors. In light of these and other concerns (described below), the manuscript needs either a substantial amount of additional data or a substantial reduction of interpreted significance of findings prior to publication.

Reviewer 1 Comments for the author
If the authors are going to make any claims regarding the “role” of H3.15, they must include phenotypic characterization of a loss-of-function mutation (preferably a null) to substantiate the claim with regards to a wild-type context. Overexpression data are extremely suspect, particularly when accompanied by data demonstrating that reversion of the amino acid of interest does not suppress the phenotype of interest.
Response: We understand the concern from Reviewer#1 and created a htr15 mutant null allele by CRISPR. The mutated sequence contained a thymine (T) insertion at the 5’ end of the HTR15 coding sequence, creating a frameshift mutation resulting in the presence of multiple premature stop codons (Fig. S5B). We found that the htr15 mutant shows smaller callus size than wild type both in wounding and CIM induction conditions (Fig. 3). Together with the opposite effect of gain of function of HTR15 we concluded that HTR15 regulates callus formation positively.

The authors have defined the promoter region as the “1952bp genomic fragment upstream of the HTR15 coding region” (line 282), but have no evidence that this region is sufficient to replicate the normal expression pattern of HTR15 in the absence of a rescue data (there are numerous examples of plant genes in which DNA sequence embedded in the first intron or the 3’ UTR are required for proper expression). At the very least, in situ data are needed to substantiate the data from the reporter lines.
Response: We understand Reviewer#1 concerns that although it is common practice to use 500 bp upstream of transcription start site to construct reporter line, there might be additional regulatory sequences. To address this point, we performed complementation assays by introducing HTR15 driven by the 1952bp genomic fragment upstream of the HTR15 coding region and found that it can restore the phenotype of htr15 to wild-type level (Figure S5C and D). We thus conclude that the promoter used in our study is adequate to analyze the expression pattern of HTR15.

Another reason the authors need a loss-of-function mutant is that the presented data from the H3.15 reporter lines are extremely suspect. qRT-PCR data (Figure 1a) reveal that the endogenous transcript level of H3.15 is increased ~2-fold at 24h and ~4-fold at 48h relative to 0h. Yet the H3.15 reporter lines indicate no expression at 0h and thus a much greater fold-change at 24h and 48h (Figure 1c).
Response: This concern is related to the previous point. Genetic complementation validated the sequence used to create a promoter to report expression pattern. Our result using qRT-PCR and the fluorescent reporter line show the same temporal dynamics but distinct amplitudes, which is common because qRT-PCR and measurement of fluorescence do not have the same dynamic ranges. To perform qRT-PCR, although we tried to collect samples at wound site as small as possible in size, it was still inevitable to harvest cells proximal to the wound site, which did not express HTR15. This would affect the average HTR15 levels. Yet, altogether our results support the main conclusion: wound causes an increase in HTR15 expression at the precise site of wounding.

As noted above, the observation that reversion of the conserved lysine residue has no effect on the overexpression callus enhancement phenotype (Figure 5) strongly detracts from the proposed model.
Response: We thank Reviewer#1 for pointing out insufficient explanation or our results that led to misunderstanding. When we performed the addition of K27 residue at HTR15 we expected that it would not restore the phenotype. However, we later realized that reports from recent biochemical studies showed that residues 28 and 29 are crucial for proper deposition of trimethylation on K27 by PRC2. (Moritz, L. E. & Trievel, R. C. Structure, mechanism, and regulation of polycomb-repressive complex 2. J. Biol. Chem. 293, 13805–13814 (2018). Since the residues 28 and 29 of HTR15 are divergent from WT within a larger region surrounding K27, it is expected that we would not observe complementation by the unique addition of K27. In contrast, substitution of aa 22-35 in H3.15-KA was sufficient to restore the phenotype in spite of remaining important substitution of K4N. This is

a strong argument to attribute the phenotype to the K27 methylation, supported further by the demonstration of the impact of H3.15 on levels of H3K27me3.
In line with this, it is striking that the authors do not address whether or not H3.15 is conserved in plants. Absence of conservation would strongly argue that the observed overexpression phenotype is indeed gain-of-function and/or that the authors are not characterizing a mechanism of plant regeneration that is generally representative of the plant kingdom (a characteristic which is used to motivate this manuscript).
Response: We thank Reviewer#1 for this important suggestion. We found that unusual H3 variants also present in the genomes of other plant species. We present data supporting a strong conservation of H3.15 amongst Brassicacaea and likely homologs in other dicots. In addition, we show how H3.15 form a group of variants distinct from the other group of most divergent H3 variants including sperm expressed H3.10 in Arabidopsis.

Additional comments:

Line 169: CAF1 does not exist to “prevent” callus formation. This is an extremely emergent phenotype resulting from loss of a key factor in chromatin assembly.
Response: We thank Reviewer#1 for pointing out this. We removed the phenotype of CAF1 mutant in the manuscript as CAF1 mutation causes pleiotropic defect on plant development and the correlation between CAF1 and H3.15 has not been investigated in our study.

Line 201: pHTR15::HTR15 should likely read 35S::HTR15 based on annotation in Figure 6.
Response: We thank Reviewer#1 for pointing out the mistake. We corrected the mistake in line 227.

Line 310: Unclear as written. Is each data point a single biological replicate with 3 technical replicates or three biological replicates with 2 technical replicates? The former would be extremely suspect, the latter would be acceptable.
Response: We thank Reviewer#1 for pointing out the unclear statement. Each data point are from three biological replicates with 2 technical replicates. We corrected the sentence in line 348.

Figure 4B: What do dotted lines indicate?
Response: in Figure 2B, we used white dotted lines to outline the area of HTR15 promoter activity. We added description of the dotted line in the legend of Figure 2B.

Figure 3D (and related): please clarify how root length determined – is this of primary explant? Please describe in some detail how measure area of callus in methods.
Response: the callus was induced from hypocotyl explant in Figure 3C and 3D. We first took pictures of explants at 28d after CIM incubation, then used image-Pro plus 6.0 software to measure the length and area of the hypocotyl explant from the pictures respectively, and then calculated the value of area/length for each explant. We added some description of the methods in Figure 3 legend from line 581 to line 582.

Figure 4B: Non-callus control is needed for this comparative analysis.
Response: We thank Reviewer#1 for this suggestion. We added western blot assay to check H3K27me3 and H3K27me1 level in non-callus tissue of WT and 35S:HTR15 in Fig. 4B.

Figure 6: These data are challenging to interpret in the absence of comparative controls. One has the sense that HTR15 is incorporated everywhere based on the use of TUB2 as comparative control in 6B and 6C. Inclusion of H3K27me3 ChIP data for these loci would be very revealing as yet another comparative control. Similarly, inclusion of H3K27me3 ChIP data for WT plants is needed for 6E to enable the reader to draw meaningfully inferences regarding the impact of overexpression of H3.15 on enrichment of H3K27me3.
Response: We thank Reviewer#1 for this suggestion. Fig. 6E showed the H3K27me3 levels between 0d and 30d in WT plants to compare the dynamics of H3K27me3 level at WOX11 locus. For Fig. 6B, 6C and 6F, we add WT as the comparative control, the relative enrichment was quantified by normalizing the amount of immunoprecipitated fragment to input DNA and then by normalizing the value for 35S::HTR15-3flag against the value for the wild type as a negative control.

Figure S1: Please clarify if panels B-E are all from 4d seedlings.

Response: They are from seedlings of different development stage. B: 7d old seedling was cut at hypocotyl, and seedling at 2 day after cutting was stained and shown; C: rosette leaf from 3 week seedlings was cut, the leaf at 4d after cutting was stained and shown; D: root of 7d old seedling was cut, the root at 1d after cutting was stained and shown; E: 7d old seedling was cut at hypocotyl, and hypocotyl at 2 day after cutting was checked by confocal imaging.

Figure S4C: What is expression relative to (y-axis)?
Response: We thank Reviewer#1 for pointing out this issue. The expression values were relative to those in 0h sample (before treatment). We added the data at 0h to the figure and added description.

Figure S7C: What are criteria for scoring "callus" at 2d?
Response: The criteria for scoring callus is the observation of at least two new cells at the wound site according to [Iwase et.al 2011, Current biology]. We have removed this panel.

Figure S6 (and accompanying analysis): There is no obvious difference in expression of reporter genes between wild-type and transformed roots. Is the implication that the authors cannot replicate the findings of the 2010 paper? If they cannot, the implications of this observation should be addressed. Otherwise, if H3.15-enabled callus does follow a different pathway, this observation would once again strongly argue that this is a gain-of-function phenotype.
Response: We understand Reviewer#1 concern. In our experiment, two kinds of callus were investigated, the wound-induced callus and CIM-induced callus. In wounding condition, callus formation did not follow root development pathway, while H3.15-mediated callus formation under CIM condition follow root pathway, which is consistent with previous reports. As H3.15 overexpression did not change the pathway, we removed these data from the manuscript.

Reviewer 2 Advance summary and potential significance to field
This paper looks at the role of the histone variant H3.15 during regeneration in Arabidopsis and suggests that H3.15 promotes reprogramming and regeneration by reducing the repressive mark H3K27me3 (unlike H3.1, HTR15 lacks a lysine at residue 27 so can not carry the H3K27me3 modification) and so facilitating the activation of key regulators such as transcription factors. There is convincing data to show that HTR15 reporters are strongly induced by wounding or growth on callus inducing media (CIM), and that induction occurs in pericycle cells in roots, which are known to be important for regeneration on CIM. This is correlative evidence for a role of HTR15 in regeneration, the functional evidence mainly rests on over expression experiments using 35S:HTR15 and 35S:HTR15-KA (a modified version where the region around residue 27 is swapped with that of canonical H3.1, potentially allowing HTR15 to be methylated at K27), the former but not the latter giving rise to larger callus after wounding or growth on CIM.

I found it an interesting paper in an area of intense research interest and the central hypothesis (that HTR15 induction leads to HTR15 incorporation into chromatin and loss of H3K27me3 mediated repression of key genes promoting regeneration) is original and appealing. The data presented is consistent with this. However I did not find the data compelling, mainly because it relies very heavily on gain of function approaches and the characterization of the materials is limited. In its present form I don't think the paper is acceptable without addressing some of the issues below.

Reviewer 2 Comments for the author
Main points
1. A very obvious omission in the paper is the use of loss of function mutants or knock down lines to test the hypothesis that HTR15 is important for regeneration. There is no mention of this anywhere in the m/s. Redundancy does not seem to be an issue as HTR15 is unique amongst the histone variants, based on the phylogeny presented and also in terms of its induction by wounding. Testing whether reducing HTR activity by loss of function mutation (or conceivably artificial microRNA etc) can interfere with regeneration would add considerably.
Response: We understand the concern from Reviewer#2 and created a null htr15 mutant allele by CRISPR. The mutated sequence contained a thymine (T) insertion at the 5' end of the HTR15 coding sequence, creating a frameshift mutation resulting in the presence of multiple premature stop codons (Fig. S5B). We found that htr15 mutant shows smaller callus size than wild type both in

wounding and CIM induction conditions (Fig. 3 new panels). Together with the opposite effect of gain of function of HTR15 we concluded that HTR15 regulates callus formation positively.

2. Another striking omission is the lack of any mention that HTR15 also lacks a lysine at residue 4, so the H3K4me2 and H3K4me3 marks that are associated with gene activity are also likely to be reduced by incorporation of HTR15, instead the focus is very specifically on H3K27me3. So it is not clear that incorporation of HTR15 would necessarily be activating and there is no attempt to look at effects on H3K4me3.
Response: We thank Reviewer#2 for this excellent suggestion. We have reported that 35S:HTR15-KA with the K4N substitution but without the K27-K31 substitution does not show the 35S:HTR15 phenotype, arguing that the K4N substitution does not have a strong impact in this context. In addition, we have performed a complementation assay by substituting N4 to K4, the construct pHTR15:HTR15-N4K still restored the htr15 phenotype to WT, suggesting that the impact of the loss of HTR15 function is not directly associated with methylation at K4.

3. The evidence that HTR15 promotes regeneration is that 35S:HTR15 over-expression lines have large callus on wounded or CIM cultured tissue, and increased expression of some important transcription factors regulating regeneration. In all cases the assays seem to be performed a long time after induction (14 and 28 days for the callus assays, 40 days for the analysis in Fig S5, 8 days for the gene expression analysis). This seems late, so that the differences could be very downstream (for example, increase in cell division) rather than necessarily a greater ability to reprogram somatic cells. It would add to show whether the induction of genes like WOX11 is more rapid and more extensive using reporter lines.
Response: We thank Reviewer#2 for pointing out that we have not explained clearly that H3.15 is involved in early steps of callus induction. Although the visual phenotype is recorded at relatively late time points for practical reasons, we observe induction of HTR15 expression within days after wounding (Fig1) and callus induction (Fig2) and we now have included the phenotype of the null allele htr15, which supports strongly the involvement of H3.15 in callus induction. In addition, we used the pWOX11:GUS reporter line to study the impact of 35S:HTR15 and report that WOX11 promoter activity is stronger in 35S:HTR15 than in that in WT at early stage during CIM incubation (Fig. S9).

4.There is very little description of the phenotype of the over expression lines. For example, western blot analysis of total histone extracts suggests that 35S:HTR15 lines have a very marked decrease in H3K27me3 levels in tissue grown on callus. Given that a large fraction of the genome is H3K27me3 decorated (at least in seedlings, it could be different in callus but probably not since the modification is readily detectable on histone blots) this would suggest that HTR15 must be replacing H3.1 at very many genes, not just at genes specifically involved in regeneration. Similarly, ChIP results using 35S::HTR15-FLAG retrieve chromatin at TUB2 at 2% of input, which might suggest that HTR15 may be widely incorporated in the genome at least in these lines. If this is the case one might expect 35S:HTR15 plants to show other phenotypes, but no description is provided of what these plants look like.

Response: We thank Reviewer#2 for this insightful suggestion. So far we did not observe other phenotypes of 35S:HTR15 except inducing callus formation upon wounding or CIM incubation. However, we did not test in detail flowering time and other physiological phenotype. These observations are in line with the western blot in Figure 4B showing that there is not a marked reduction of levels of H3K27me1/3 before induction of callus. This contrast with the strong reduction of H3K27me3 levels observed 30D after induction in 35S:HTR15 background. These observations suggests that a small fraction of H3.15 produced in 35S:HTR15 plants is incorporated before callus induction, in agreement with the lack of vegetative phenotype in these plants. H3.15 also show substitutions in position 87 and 90, which are critical for incorporation by chaperones HIRA and CAF1. So we hypothesize that H3.15 only incorporates into nucleosomes under conditions of lower availability of H3.1 or H3.3, which might take place at appropriate steps during callus induction and formation.
For H3.15 enrichment, we added WT as the comparative control, and repeated the ChIP assay, confirming that H3.15 was enriched at the WOX11 locus.

5. Another problem with the over expression lines is that HTR15 is now present at high levels in many cell types where it is not normally induced. So even if regeneration is facilitated it is not

clear that this necessarily reflects the wild type function (in which the induction seems more specific to pericycle).
Response: We understand the concern of Reviewer#2 that stems largely from the lack of analysis of a null allele. But this data is now provided and supports the role of HTR15 in callus formation. Although HTR15 is present at high levels in many cell types in overexpression line, we speculate that it may only incorporate into nucleosome at certain cell types or under certain conditions. But we hope that Reviewer#2 would agree that to demonstrate this is particularly challenging and beyond the scope of this study.

Minor points

There is no expression analysis for example to show that the transgenic lines giving a regeneration phenotype do have increased HTR15 levels.
Response: We thank Reviewer#2 for this suggestion. We added the RT-qPCR analysis of HTR15 overexpression lines that show the increased expression of HTR15 as expected (Fig. S5A).

The 35S:HTR15-FLAG line used for ChIP is very little characterised. Some kind of complementation analysis or even western blot to show expression of the fusion protein is usually included. In the ChIP experiments (Fig 6 B, C, F) there is an important control missing, i.e. using a non transgenic line for comparison in order to give confidence that the antibody is specifically pulling down HTR15-FLAG chromatin.
Response: We thank Reviewer#2 for this suggestion. We added RT-qPCR and western blot assay to characterize the 35S:HTR15-FLAG line in Fig. S7. For ChIP experiments, we added WT as the comparative control, the relative enrichment was quantified by normalizing the amount of immunoprecipitated fragment to input DNA and then by normalizing the value for 35S::HTR15-3flag against the value for the wild type as a negative control, and revised Fig. 6B, 6C and 6F accordingly.

Figure 2B 48 hour samples – what do the white dotted lines indicate?
Response: We used white dotted lines to outline the area of HTR15 promoter activity. We added description of the dotted line in the legend of Figure 2B.

Figure 4C shows relative expression but does not indicate relative to what. Uninduced samples?
Response: We thank Reviewer#2 for pointing out this issue. In Figure S4C, the expression levels were relative to those in 0h sample (before treatment). We added description in the legend.

Figure 3B and D – why is one graph shown as a box plot and whiskers, the other as a bar chart?
Response: in Figure 3B we used box plot to show the distribution of callus area, as bar chart may omit display of some individual data points; Figure 3D showed the ratio of callus area to explant length, it showed relatively low dispersion, so we just used bar chart to display the data.

Figure S7 – in part A it would be helpful to describe the stains, i.e. is the EdU Blue or pink, the latter presumably but helpful to point out and describe what the blue stain is. The text states that A and B support DNA replication at 24 hours, but I could not see induction of CYCB1;1 GUS until 72 hours? In part C it is claimed that fas2-4 has a different effect on callus induction to HTR15 OE, but the assay (callus formation rate %) is a different one to what was used for the HTR15 lines, so it is not clear the comparison is valid.
Response: We thank Reviewer#2 for this suggestion. We added description of Edu staining. The nuclei were labeled with Hoechst 33342 and visualized as blue dot (425-475nm), while Nuclei with newly synthesized DNA were labelled by EdU and visualized as red dot (663-738nm).
We removed the phenotype of CAF1 mutant in the manuscript as CAF1 mutation causes pleiotropic defect on plant development and the correlation between CAF1 and H3.15 has not been investigated in our study.
For the induction of CYCB1;1, we found that CYCB1;1-GUS signal was not visible until 48h after wounding. So we replaced 72h staining image with 48h staining image in Fig. S6B. As CYCB1;1 functions as an effector of growth control at G2/M, it might be activated at a time point after DNA replication.

Figure S6 WOX5 and SCR reporter expression. It would be helpful to indicate which is the wounded (the ends?) and which the unwounded (the middle?) tissue. It was very unclear what this figure adds

really, it does not seem to show any difference between wild type and HTR OE line and the differences between wounded and unwounded tissue don't add much since not followed up.
Response: We thank Reviewer#2 for pointing this issue As H3.15 overexpression did not change the expression pattern of WOX5 and SCR, we removed these data from the manuscript.

Reviewer 3 Advance summary and potential significance to field
A deep characterisation of the role of HISTONE THREE RELATED 15 (H3.15) during early wounding response and CIM-induced callus formation, in Arabidopsis hypocotyl and root tissues.
In summary, the manuscript offers novel data about H3.15 expression profile, with involvement of auxin in its expression regulation, the phenotypic effects of its ectopic expression, its roles on the regulation of H3K27 methylation, specifically for and on the expression of WOX5, WOX11, LBD16, LBD18, LBD29, and ERF115 possibly via direct H3K27 methylation.
These results are significant and of general interest for advancing our understanding of wound response and auxin-induced callus formation during in vitro, CIM-induced, regeneration.
Particularly interesting and relevant for a broad audience of developmental biologists is the clear link with epigenetic regulation.

Reviewer 3 Comments for the author
I believe this manuscript is suitable for publication, but I suggest minor revisions (below).

In general, the manuscript reads quite dense and the sequence of experimental results could be paced with more explicit rationales, but this is left to the personal styles of the authors. I would simply suggest the authors to see if there is room for smoother transitions from one experiment to another.
Response: We thank Reviewer#3 for the support and have added some sentences in the results section to smooth transitions between experiments which will help the reader.

More specific comments for minor revisions:

- Lines 105-107 and Fig1.
Isn't Fig1 about early response to wounding, without CIM? If so, I don't see why mentioning callus at all. Is it visible in Fig1? Also, in Fig 1 it should more clear what is the difference between panels C, D, E. Finally, it would help to know more about the "wounding assay": where (meristem, elongation zone, etc) and how is the wounding generated?
Response: We thank Reviewer#3 for pointing this fact that required clarification. Fig. 1 shows the HTR15 response to wounding, not to CIM. In Fig. 1C, the callus is visible at 3d and 4d, HTR15 is also expressed in these calluses.
Fig. 1C shows progressive induction of HTR15 expression during wounding response from 0h to 4d. Figures 1D and 1E select one time point (24h after wounding) and used higher magnification to show that HTR15 is expressed in pericycle.
For the wounding assay, 7d old seedlings were cut at the middle of root (elongation zone), the lower parts were subjected to confocal imaging. We added description of the wounding assay in Fig. 1A legend from line 552 to line 555.

- Lines 155-158 and Fig S6
It is unclear in S6 that the fate-specific expressions of WOX5 and SCR are different in 35S::HTR15 vs wt. Also, even if this were true, why excluding the possibility that only those cells expressing WOX5 and SCR will participate to callus formation, either in WT or in 35S::HTR15?
Overall, the argument about a separate developmental pathway seems weak and unconvincing.

Response: We agree with Reviewer#3. As H3.15 overexpression did not change the expression pattern of WOX5 and SCR, we removed these data from the manuscript.

Second decision letter

MS ID#: DEVELOP/2019/184895

MS TITLE: The atypical histone variant H3.15 promotes regeneration in Arabidopsis thaliana

AUTHORS: An Yan, Michael Borg, Frederic Berger, and Zhong Chen

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors have done an admirable job of responding to previous concerns regarding the manuscript. In particular, the generation and characterization of the hta15 mutant strengthens the manuscript considerably.
Some of the major claims made by the authors, however, are still primarily based on phenotypes associated with overexpression. As a result, new data need to be included (as detailed below) or a number of claims need to be adjusted accordingly.

Comments for the author

The authors now show that loss of HTR15 results in a modest defect in callus formation. These data are critical, as noted by earlier reviewers, and the inclusion of data demonstrating rescue of the callus phenotype of htr15 explants with HTR15:HTR15 is also a strong addition. However, all of the characterization of H3K27me1, H3K27me3, gene expression, and chromatin association are based only on 35S dependent data and so still quite possibly gain of function. The authors need to either repeat the analyses in figures 4, 5, and 6 (specifically 4B, 4C, 5D, 6A, 6D, 6E) using wild-type versus htr15 lines (thus demonstrating that some aspect of gene expression and/or H3K27me1/3 is dependent on endogenous HTR15) or categorically acknowledge throughout the document that these data are derived from plants in which HTR15 is overexpressed and are unsubstantiated by loss-of-function data.

Additional comments:

Lines 32-4 There are no data presented that “demonstrate” that “H3.15 deposition facilitates removal of the transcriptional repressive mark H3K27me3” or that it is this deposition that “leads to transcriptional derepression”. This section of the abstract should be removed or greatly softened.

Line 141 Suggest changing “H3.15 is sufficient to promote wound induced callus formation.” to “H3.15 is sufficient to promote wound induced callus formation in this context.”

Line 198 With regards to sufficiency, the authors are strongly encouraged to explicitly note that these data also demonstrate that overexpression of H3.15 is not sufficient to detectably affect H3K27me1 and H3K27me3 in plant tissue prior to CIM induction. This result should also be incorporated within any postulated model (Fig S11 implies sufficiency).

Line 201 The authors do not demonstrate that endogenous H3.15 is incorporated in chromatin and only have the correlation that detectable association of overexpressed H3.15-FLAG is accompanied

by reduced levels of H3K27me1/3 and similarly increased expression of genes (which itself is strongly associated with reduced levels of H3K27me3). Here and throughout the manuscript, the authors need to substantially soften the claim that they have "demonstrated" that it is the incorporation of H3.15 that is the proximal cause of reduced H3K27me3 and etcetera. To be clear, I think it is fine for the authors to discuss such a model in the discussion based on the presented data, but only if they acknowledge that it lacks support from loss-of-function plants.

Line 227. There are no data demonstrating incorporation of H3.15 in tissue when gene expression is assayed (8d).

Line 239 "these data"

Line 274 The presented data do not demonstrate that incorporation of H3.15 is independent of replication only that expression of HTR15 occurs prior to replication. The authors should either greatly soften or remove all claims with regards to timing of potential HTR15 incorporation (particularly in absence of data that endogenous HTR15 is incorporated).

Figure 3 Based on the appearance of the tissue, is there any chance that panels C and E are reversed? The length of incubation is unclear in panels D and F and the reason for switching the length of incubation for different panels is unclear. This variation in incubation time becomes particularly troublesome when attempting to compare results from different figures. The authors are strongly advised to select a one or two times and use those times consistently, or specifically address why different experiments necessitate different times.

Figure 4. Based on the authors' model, induction of endogenous H3.15 in wild-type plants should result in reduced H3K27me1/3 on CIM. They should examine this possibility. Further, as noted above, examination of this possibility in both wild-type and htr15 plants would substantially enhance this manuscript.

Figure 5 Here the samples are now 30d. Do the authors really only have two biological replicates for panel E?
Is there any reason the authors did not examine H3K27me1 in panel D as they did in Figure 4?
Figure 6. What is the incubation time for panel B? Please specify 35S:H3.15-FLAG here and throughout. The value of panels 6E and 6F is unclear, particularly in the absence of a comparative wild-type control. It has previously been indicated that H3K27me3 enrichment is decreased in callus tissue relative to non-callus tissue. Observing the same in 35S:H3.15 plant is unsurprising in the absence of demonstrating that the decrease is enhanced relative to wild-type plants – thus wild-type plants are needed as a control here. The proposed effect of H3.15 incorporation would be much more convincing if it were observed at a locus in which expression was unchanged and yet H3K27me3 was reduced.

Figure S5 Given the claims based on presented analysis of 35S:HTR15-KA, it is strongly advised that the authors also include HTR15:HTR15-KA in panels C and D to demonstrate that this construct is not able to rescue htr15 callus formation when expressed at presumed wild-type levels.

Figure S9. The authors should specify if these loci are significantly differentially enriched for H3K27me3 rather than relying on visual images.

Reviewer 2

Advance summary and potential significance to field

The authors have added extra data and improved the paper. I think they have done enough to satisfy my initial comments and it is an interesting paper that is suitable for publication in development now.

Comments for the author

The S data figure showing relative expression of the HTR15 over expression lines should indicate relative to what (non transgenic?) as a minor comment.

Reviewer 3

Advance summary and potential significance to field

A deep characterisation of the role of HISTONE THREE RELATED 15
(H3.15) during early wounding response and CIM-induced callus formation, in Arabidopsis hypocotyl and root tissues.
In summary, the manuscript offers novel data about H3.15 expression profile, with the involvement of auxin in its expression regulation, the phenotypic effects of its ectopic expression, its roles on the regulation of H3K27 methylation, specifically for and on the expression of WOX5, WOX11, LBD16, LBD18, LBD29, and ERF115 possibly via direct H3K27 methylation.
These results are significant and of general interest for advancing our understanding of wound response and auxin-induced callus formation. The clear link with epigenetic regulation is particularly interesting and relevant for a broad audience of developmental biologists.

Comments for the author

The manuscript has improved and the most important issues I raised have been addressed. However, there are still a few minor points that in my opinion should be clarified.

Fig1C:
I don't see any convincing sign of callus formation, which does not surprise me since you are not adding any CIM in these experiments. I would suggest you either justify your statements about callus (maybe with a marker), and possibly add an arrow in the figure, or just remove the callus mention altogether in this set of experiments. It is not that important for your argument, anyway. Also, can you specify in the text how many roots you looked at?

Fig 1C legend:
It is still largely unclear where the cut has been performed. This is important in order to compare these results with existing studies on wounding and regeneration.
To this end, the authors should avoid using generic and imprecise statements such as "middle of the root" and "lower parts". Use instead objective descriptions such as "xx microns from the tip or from the meristem/elongation transition zone" and "proximal" or "distal".

Title:
In my previous review, I missed an important point about the proposed title. Claiming H3.15 "promotes regeneration" based on induced callus formation and expression of WOX11 is an exaggeration that would confuse the reader. This work does not show any proper regeneration, either at the morphological or molecular level. Callus formation, or the identification of a few downstream genetic targets, does not justify the claim of promoting actual regeneration. Regeneration of what tissue, what cell types? Fig 3 and Fig 5 are about callus formation, not regeneration.
For the same reason, the Methods paragraph "Regeneration assay" has a misleading title.

Second revision

Author response to reviewers' comments

[Response to Reviews is also included as a formatted PDF file in Supplementary Information]

Reviewer 1 Advance summary and potential significance to field
The authors have done an admirable job of responding to previous concerns regarding the manuscript. In particular, the generation and characterization of the hta15 mutant strengthens the manuscript considerably.
Some of the major claims made by the authors, however, are still primarily based on phenotypes associated with overexpression. As a result, new data need to be included (as detailed below) or a number of claims need to be adjusted accordingly.

Reviewer 1 Comments for the author
The authors now show that loss of HTR15 results in a modest defect in callus formation. These data are critical, as noted by earlier reviewers, and the inclusion of data demonstrating rescue of the callus phenotype of htr15 explants with HTR15:HTR15 is also a strong addition. However, all of the characterization of H3K27me1, H3K27me3, gene expression, and chromatin association are based only on 35S dependent data and so still quite possibly gain of function. The authors need to either repeat the analyses in figures 4, 5, and 6 (specifically 4B, 4C, 5D, 6A, 6D, 6E) using wild-type versus htr15 lines (thus demonstrating that some aspect of gene expression and/or H3K27me1/3 is dependent on endogenous HTR15) or categorically acknowledge throughout the document that these data are derived from plants in which HTR15 is overexpressed and are unsubstantiated by loss-of-function data.
Response: We thank Reviewer#1 for the supporting comments and for pointing out these issues. Extending analysis to htr15 mutants would be far beyond the scope of the revisions and also will likely be difficult due to the low amount of callus available in the mutant line. We have thus chosen the alternative solution proposed by reviewer#1 and adjusted some claims in the manuscript and precised the limitations of certain conclusions in Line35, Line216, Line253, Line302, and Line 314. We must also state that 6E (now 6D) is based on analyses in WT plants, not in 35SHTR15-3XFLAG Lines but this was not mentioned clearly and is now also corrected.

Additional comments:

Lines 32-4 There are no data presented that “demonstrate” that “H3.15 deposition facilitates removal of the transcriptional repressive mark H3K27me3” or that it is this deposition that “leads to transcriptional derepression”. This section of the abstract should be removed or greatly softened.
Response: We thank Reviewer#1 and have revised the sentence based on the suggestion.

Line 141 Suggest changing “H3.15 is sufficient to promote wound induced callus formation.” to “H3.15 is sufficient to promote wound induced callus formation in this context.”
Response: We thank Reviewer#1 and have revised this sentence accordingly.

Line 198 With regards to sufficiency, the authors are strongly encouraged to explicitly note that these data also demonstrate that overexpression of H3.15 is not sufficient to detectably affect H3K27me1 and H3K27me3 in plant tissue prior to CIM induction. This result should also be incorporated within any postulated model (Fig S11 implies sufficiency).
Response: We thank Reviewer#1 and have revised the statement in line 216. The postulated model in Fig. S11 illustrates the role of H3.15 under the conditions of wounding and auxin treatment, that is, only in wounding or high auxin environment, H3.15 may regulate cell fate reprogramming through affecting H3K27me3. Without the upstream conditions, H3.15 is not sufficient to promote callus formation.

Line 201 The authors do not demonstrate that endogenous H3.15 is incorporated in chromatin and only have the correlation that detectable association of overexpressed H3.15-FLAG is accompanied by reduced levels of H3K27me1/3 and similarly increased expression of genes (which itself is strongly associated with reduced levels of H3K27me3). Here and throughout the manuscript, the authors need to substantially soften the claim that they have “demonstrated” that it is the incorporation of H3.15 that is the proximal cause of reduced H3K27me3 and etcetera. To be clear, I think it is fine for the authors to discuss such a model in the discussion based on the presented data, but only if they acknowledge that it lacks support from loss-of-function plants.
Response: We thank Reviewer#1 and have revised the sentence and removed the statement of “H3.15 incorporation” in Line 216 accordingly.

Line 227. There are no data demonstrating incorporation of H3.15 in tissue when gene expression is assayed (8d).
Response: We thank Reviewer#1 and have revised this sentence based on the comment.

Line 239 “these data”
Response: We thank Reviewer#1 and have rectified the error.

Line 274 The presented data do not demonstrate that incorporation of H3.15 is independent of replication, only that expression of HTR15 occurs prior to replication. The authors should either greatly soften or remove all claims with regards to timing of potential HTR15 incorporation (particularly in absence of data that endogenous HTR15 is incorporated).
Response: We removed the claim and will perform further studies to test further the mode of incorporation of H3.15.

Figure 3 Based on the appearance of the tissue, is there any chance that panels C and E are reversed? The length of incubation is unclear in panels D and F and the reason for switching the length of incubation for different panels is unclear. This variation in incubation time becomes particularly troublesome when attempting to compare results from different figures. The authors are strongly advised to select a one or two times and use those times consistently, or specifically address why different experiments necessitate different times.
Response: We understand the concerns expressed by reviewer #1. The different durations of treatment were dictated by experimental constraints as explained. Panel D is the quantitative analysis of callus formation data which is illustrated in panel C; panel F is the quantitative analysis of callus formation data illustrated in panel E. panels C show callus developed from hypocotyl explant, while panels E show callus developed from root explant, we found that root-derived callus formation was slower than hypocotyl-derived callus formation, which may result from different amount of starting materials. So we incubated root explant on CIM for a longer period (40 days) than hypocotyl explant (28 days). We have added explanation in line 159.

Figure 4. Based on the authors’ model, induction of endogenous H3.15 in wild-type plants should result in reduced H3K27me1/3 on CIM. They should examine this possibility. Further, as noted above, examination of this possibility in both wild-type and htr15 plants would substantially enhance this manuscript.
Response: We quantified the relative intensity of H3K27me1/3 and re-calculated the results by normalizing the value in CIM-induced callus (40d) against that in non-callus tissue (0d). we found that H3K27me1/3 levels were reduced when compared wild type at 40d with that at 0d, which means the reduction of H3K27me3 level is associated with induction of endogenous HTR15 during CIM incubation (Fig. 2). Thus, we modified Fig 4C to show the result.

Figure 5 Here the samples are now 30d. Do the authors really only have two biological replicates for panel E? Is there any reason the authors did not examine H3K27me1 in panel D as they did in Figure 4?
Response: For WB in Fig 5D and 5E, we have done two biological replicates by checking the four samples in one blot simultaneously. Additionally, we also loaded WT with 35S:HTR15-KA or 35S:HTR15-H27K separately in different blots, the results showed similar pattern with that in Fig. 5D.Hence, we quantified the WB signal from a third replicate and revised Fig. 5E based on three biological replicates.
We also checked H3K27me1 in the experiment, the H3K27me1 pattern was similar as H3K27me3. As the data in Fig 5DE were provided as supplementary information at the beginning of submission and the conclusion focused on H3K27me3, so we didn’t show results of H3K27me1. Now we added the results in the Fig. 5D and 5E.

Figure 6. What is the incubation time for panel B? Please specify 35S:H3.15-FLAG here and throughout. The value of panels 6E and 6F is unclear, particularly in the absence of a comparative wild-type control. It has previously been indicated that H3K27me3 enrichment is decreased in callus tissue relative to non-callus tissue. Observing the same in 35S:H3.15 plant is unsurprising in the absence of demonstrating that the decrease is enhanced relative to wild-type plants – thus wild-type plants are needed as a control here. The proposed effect of H3.15 incorporation would be much more convincing if it were observed at a locus in which expression was unchanged and yet H3K27me3 was reduced.

Response: We thank Reviewer#1 and we are sorry about the lack of mention that experiments reported in E were done in WT background and directly reflect published data shown in FigS9. This is now clearly indicated in the legend and text and we have changed the flow of the text and figure panel order to provide more clarity.
Hypocotyl explants were cultured on CIM for 30 d for panel B and C.
For panels 6E, now changed to 6D, firstly the value of immunoprecipitated fragment was normalized to input DNA for each sample, then the value for 30 d CIM sample was normalized against that for 0 d explants. Both samples are wild type.
For panels 6F, now changed to panel 6E, firstly the value of immunoprecipitated fragment was normalized to input DNA for each sample, then the value for 30d CIM 35S::HTR15-3flag explants was normalized against that for wild-type seedlings, and the value for 0d CIM 35S::HTR15-3flag explants was also normalized against that for wild-type. Wild type was used as the comparative control.
In spite of overexpression of HTR15, there is obviously no incorporation of H3.15 at WOX11 in absence of callus induction. This is an important point because it suggests that H3.15 incorporation is triggered by a specific mechanism, linked to CIM treatment at genes that respond to the treatment. So we do not agree with the suggestion from Reviewer#1 to monitor H3K27me3 levels at random sites which are not affected by callus induction. During CIM incubation, both 35S:HTR15 and WT showed decreased H3K27me3 enrichment, but 35S:HTR15 show lower levels of H3K27me3 at WOX11 locus compared with WT (Fig. 6D now 6F), suggests the decrease of H3K27me3 is enhanced in 35S:HTR15. Although we didn't check H3K27me3 enrichment at WOX11 locus in 35S:HTR15 before CIM incubation, our western blot results suggest that H3K27me3 levels of 35S::HTR15 are similar to wild type before CIM induction, so we believe that the decrease of H3K27me3 during callus formation is enhanced in 35S:HTR15, based on results in Fig 6D (now 6F).

Figure S5 Given the claims based on presented analysis of 35S:HTR15-KA, it is strongly advised that the authors also include HTR15:HTR15-KA in panels C and D to demonstrate that this construct is not able to rescue htr15 callus formation when expressed at presumed wild-type levels.
Response: We thank Reviewer#1 for the suggestion. However, this would require preparing new construct of pHTR15:HTR15-KA and transforming into htr15 will be time-consuming, we will prepare this line and test it in our future work. This is clearly beyond the scope of the time required for revisions.

Figure S9. The authors should specify if these loci are significantly differentially enriched for H3K27me3
rather than relying on visual images.
Response: We are sorry that reviewer#1 missed a piece of information. FigS9 is based on published data to suggest the experiment using ChIP Q PCR that quantifies directly the levels of H3K27me3 at the WOX11 locus in callus versus somatic tissues prior CIM application. Our data showed that levels of H3K27me3 enrichment at the WOX11 locus are reduced in callus versus somatic tissue prior induction (Fig. 6D). This result is consistent with previous ChIP-chip assay reported in Figure S9.

Reviewer 2 Advance summary and potential significance to field
The authors have added extra data and improved the paper. I think they have done enough to satisfy my initial comments and it is an interesting paper that is suitable for publication in development now.

Reviewer 2 Comments for the author
The S data figure showing relative expression of the HTR15 over expression lines should indicate relative to what (non transgenic?) as a minor comment.
Response: Thank you for the comments. The expression level of HTR15 in overexpression lines were shown relative to wild type. we have added description in the legend of Fig. S5C.

Reviewer 3 Advance summary and potential significance to field
A deep characterisation of the role of HISTONE THREE RELATED 15 (H3.15) during early wounding response and CIM-induced callus formation, in Arabidopsis hypocotyl and root tissues.
In summary, the manuscript offers novel data about H3.15 expression profile, with the involvement of auxin in its expression regulation, the phenotypic effects of its ectopic expression, its roles on

the regulation of H3K27 methylation, specifically for and on the expression of WOX5, WOX11, LBD16, LBD18, LBD29, and ERF115 possibly via direct H3K27 methylation.
These results are significant and of general interest for advancing our understanding of wound response and auxin-induced callus formation. The clear link with epigenetic regulation is particularly interesting and relevant for a broad audience of developmental biologists.

Reviewer 3 Comments for the author
The manuscript has improved and the most important issues I raised have been addressed. However, there are still a few minor points that in my opinion should be clarified.

Fig1C:
I don't see any convincing sign of callus formation, which does not surprise me since you are not adding any CIM in these experiments. I would suggest you either justify your statements about callus (maybe with a marker), and possibly add an arrow in the figure, or just remove the callus mention altogether in this set of experiments. It is not that important for your argument, anyway. Also, can you specify in the text how many roots you looked at?
Response: Thank you for pointing out this issue. We have added arrows in Fig 1C to indicate the sign of callus formation. For the confocal imaging, we have checked at least 20 seedlings at each time point. This is now specified line 595.

Fig 1C legend:
It is still largely unclear where the cut has been performed. This is important in order to compare these
results with existing studies on wounding and regeneration.
To this end, the authors should avoid using generic and imprecise statements such as "middle of the root" and "lower parts". Use instead objective descriptions such as "xx microns from the tip or from the meristem/elongation transition zone" and "proximal" or "distal".
Response: We apologize for not having given sufficient precision. The roots of 7d-old seedlings were cut at approximately 2 cm (elongation zone) from the root-hypocotyl junction. We revised the description of wounding treatment in line 592-594.

Title:
In my previous review, I missed an important point about the proposed title. Claiming H3.15 "promotes
regeneration" based on induced callus formation and expression of WOX11 is an exaggeration that would confuse the reader. This work does not show any proper regeneration, either at the morphological or molecular level. Callus formation, or the identification of a few downstream genetic targets, does not justify the claim of promoting actual regeneration. Regeneration of what tissue, what cell types? Fig 3 and Fig 5 are about callus formation, not regeneration.
For the same reason, the Methods paragraph "Regeneration assay" has a misleading title.
Response: We thank Reviewer #3 for pointing this out and for the sake of clarity and precision we replaced the term "regeneration" with "callus formation" in title and methods, and some other place in the manuscript.

Third decision letter

MS ID#: DEVELOP/2019/184895

MS TITLE: The atypical histone variant H3.15 promotes callus formation in Arabidopsis thaliana

AUTHORS: An Yan, Michael Borg, Frederic Berger, and Zhong Chen
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have done an admirable job of addressing previous concerns.

Comments for the author

Minor edits:
Line 231: “promotes transcription” rather than “promotes transcriptions”
Line 308: “it will be of interest” rather than “it will interest”

Reviewer 2

Advance summary and potential significance to field

I think the authors have made a lot of effort to address the comments raised by reviewers and I think it is an interesting paper that merits publication.

Comments for the author

Figure 4A presents a phylogenetic tree but with no explanation (what kind of analysis, are the values bootstrap values, how many runs etc) in legend or in methods.

Figure S7 presents alignments of H3.10 and H3.15 proteins from various species. however, for H3.15 the proteins are all from Brassicaceae which hardly argues for broad conservation. Is H3.15 conserved outside this family and more broadly? The text (lines 190 - 192) suggests there is a homologue in Medicago, but the figure only shows this for H3.10 and not for H3.15. The authors should either be more open, and acknowledged that HTR15 is not widely conserved, or should replace Figure S7 with a better one to show broader conservation in dicots (asterids, rosids, basal groups including Amborella etc)

Reviewer 3

Advance summary and potential significance to field

A deep characterisation of the role of HISTONE THREE RELATED 15 (H3.15) during early wounding response and CIM- induced callus formation, in Arabidopsis hypocotyl and root tissues.
In summary, the manuscript offers novel data about H3.15 expression profile, with the involvement of auxin in its expression regulation, the phenotypic effects of its ectopic expression, its roles on the regulation of H3K27 methylation, specifically for and on the expression of WOX5, WOX11, LBD16, LBD18, LBD29, and ERF115 possibly via direct H3K27 methylation.
These results are significant and of general interest for advancing our understanding of wound response and auxin- induced callus formation. The clear link with epigenetic regulation is particularly interesting and relevant for a broad audience of developmental biologists.

Comments for the author

The manuscript is significantly improving, but still requires minor clarifications.
There are only two minor point still outsdtanding, in my opinion. I am sorry to keep picking on it, but I would like to avoid mis-interpretations in fig 1C.
I still see no clear evidence that the cells indicated by the arrow in fig 1C are indeed part of callus tissue. The authors also write on line 106-107 "in proliferating callus cells (Fig. 1C and Fig. S1A)": I see no evidence of proliferation, either (no cell division marker is used, for example). Is it possible that these cells are cells pre-existing the cut (so without cell divisions occurring) that are simply expanding? If these should be called "callus" or not, it becomes a bit semantic, but the authors should discuss this point more carefully.

Also, the position of the cut is now described as "The roots of 7d-old seedlings were cut at approximately 2 cm (elongation zone) from the root-hypocotyl junction": besides the fact that is somehow unusual to indicate a position on the root through the distance from the root-hypocotyl junction (itself hard to define and pinpoint anatomically) rather than from the root tip, I am not sure how the authors can conclude it is "elongation zone" and not "differentiation zone". If this is an important point for their argument, this should be reported more carefully, for example examinining uncut, 7d-old seedlings under confocal and showing that on average 2cm from hypocotyl falls in the elongation zone.