Dear members of the editorial board.

We highly appreciate that the editorial board considers to publish our data in Plant Direct and the opportunity to further improve the manuscript by providing helpful and constructive comments from the chosen reviewers.

The specific concerns that need to be addressed to meet the Plant Direct criteria are:

(1) Both Reviewers suggest to provide quantification of cell images and provide sample size

Done as requested (see below). The amount of the samples analyzed are added to the figure legends.

(2) Reviewer #1 ask for clarification of microscopic method.

The result and method part have been extended and updated. Since the editorial board demands to add important methodical detail to the text part, we removed the Sup. Fig S1 and S2 and designed a new Fig. 10, now integral part of the manuscript.

(3) The concern over image quality needs to be addressed.

We have done this extensively. Please see our detailed comments to reviewer #1 and the updated manuscript text in the corresponding result and method part.

Thus, we hope that these three major concerns were satisfactory addressed. For detailed comments addressing point by point see below.

Reviewer comments:

Reviewer #1:

In general, I think that the authors have been able to show the asymmetric distribution of EHB1. Especially in figures 1, 3 and 7. However, I am not convinced by their claim about the effect of BFA. With the poor image quality in this figure (Fig. 5), without quantification, I don't think we can draw that conclusion. The chemical treatments of CHX, CYD, BFA and NPA are also extremely harsh and this can be seen on their very strong effects on gravitropism. The authors should reconsider their conclusion of the differential effect of BFA or present better microscopy data.

Concerning the poor quality of the presented confocal images, we would like to clarify that the purpose of our investigation consisted in documenting the asymmetric distribution of GFP-EHB1 after and during an extended gravitropic stimulus, i.e. demonstrating concentration differences of GFP-EHB1 in the upper and lower parts of a horizontally placed root (Fig. 1 F). To determine fluorescence intensity differences with a minimum of optical artifacts we developed an “end-on” approach for confocal microscopy that allows a mostly error free determination of the fluorescence-intensity ratios (top/bottom) in the root tip. “Error free” in this context means that despite the tissue related scatter associated with confocal microscopy, the upper and the lower zones of interest (ROIs) possess - because of identical optical distances from the root apex (e.g. Fig. 1 F and Fig. 3) - the same scatter-generated fluorescence loss. With confocal microscopy in the common configuration (objective perpendicular to the root axis) one could, of course, also determine the asymmetry of EHB1-GFP fluorescence intensities in a cross-section view. In this case, however, when reconstructing the optical X-Z-plane, the scatter along the Z-axis (top/bottom) would become unequal such that the fluorescence intensities of GFP-EHB1 at top and bottom would be affected. The net result is an error prone change of the real spatial asymmetry of GFP-EHB1 in the root cross-section which would be hard to correct (e.g. by calculating or measuring scatter increases along the Z-axis). Thus, we developed the applied

end-on technique, which is optimized for quantifying fluorescence-intensity differences (top/bottom) in the root cross-section at the cost of diminished optical resolution. Of course, the configuration commonly employed in confocal microscopy achieves a superior optical resolution in optical layers close to the objective, but, as mentioned above, the important top/bottom fluorescence-intensity differences are more prone to scatter artefacts, particularly, when one has to cope with unusually large optical depths of up to 120 µm as in our experiments (Figs. 1 F and 3). In the revised version of the manuscript we have included these elaborations (with similar wording) in the early parts of the result and discussion part.

Leaving these technical matters aside, we would like to emphasize that we think that BFA effect on EHB1 on tissue level is significant for which reason we consider these data as important for our model (see our comments to the previous Fig. 5 below).

There is another issue with the microscopy method. I get the impression that during imaging the seedling is actually growing with the root upwards and shoot downwards. The authors state that the seedling is oriented vis-à-vis with the objective. In the methods it reads that an upright microscope was used, so again this tells me that the seedling is growing inverted. Please can the authors clarify this, since the wording and procedure is somewhat complicated and it's written down in a manner that confuses me slightly.
If the seedlings are indeed inverted when microscopic images were taken, would that not affect the gravitropic response during imaging? Would it not be better to use an inverted microscope, since then the seedling would be actually oriented with shoot upwards and root downwards? Please discuss this issue.

We have rephrased the respective chapter in the manuscript and tried to clarify the necessity of the applied end-on microscopy. Regarding inverse microscopy: As rightfully noted, end-on microscopy (as we utilized it) results in inverting the seedling. Unfortunately we did not have access to an inverted microscope in our facility. Although, end-on analysis as we did it and analysis using an inverted microscope would somehow create the same problems but, of course, would make our work much easier. Concerning the scientific outcome: We observed first visible EHB1-GFP redistribution after 5-10 minutes. The time to acquire images was in any case less than 3 minutes. We tried to minimize this problem by shortening the scanning period as much as possible (see above) while choosing a long lasting horizontal g-stimulus.

General comment about the microscopy images: The quality of them is not great and although in most cases we can see asymmetric distributions, what I would like to know is how many independent experiments and seedlings do these images represent? Since we have no quantification for most of these figures I think this is the bare minimum info required. In Fig. 7 the authors show how it should be done, with a time-series, quantified, over multiple samples.

We added information about the number of investigated roots in the material and methods part. Especially for the chemical treatments these data were (as requested) quantified and subsumed in an additive column chart (now new Fig. 7)

Fig. 1 G-J: How do we know this is not just autofluorescent signal? This signal seems extremely vague.

Compared with the EHB1-GFP fluorescence the fluorescence of AGD12-GFP and the GFP-control (obtained by the Schülling lab) were considerably weaker. However, we evaluated the AGD12-GFP fluorescence signal by applying a λ-scan, which allows to analyze the emission for genuine GFP-fluorescence. We added the respective analyses to the supplemental material (see Sup. Fig. S2).

Fig. 4: The quality of the images in figure 4 is lacking and I'm not sure we can conclude anything from them.

As mentioned above, quality of the images depends on optical conditions of the root tissue in our methodological approach and cannot be improved without endangering the dynamics of the EHB1 redistribution. As mentioned we intent to show that EHB1 gets redistributed once the root tip is orientated into a vertical position. To make this outcome more convincing, we added (as done in Fig. 5 and the new Fig. 7) a graph/column chart of the analyzed root tips comparing the two ROIs over time.

Fig. 5: A control image is lacking

We think that Fig 1 E & F would be sufficient as controls for Fig. 5, since the experimental setup was similar to Fig. 1.

Fig. 5: E-F I am not sure we can conclude anything about the effect of BFA on EHB1-GFP from this image alone.

First of all, to our great regret, we have to admit that some of the images in Fig. 5 have been mixed up by a last-minute rearrangement. Therefore, the comments in the results and discussion section did not match some of the pictures given. This discrepancy was also noticed by reviewer #2 (see below). We are sorry about this.

Addressing the reviewer's concerns about our conclusions of the effect of BFA: We only claim that in presence of BFA root tip remains essentially unaffected, regardless, if a gravitropic stimulus was given or not. At the moment we cannot provide experimental data, which would help us to understand the molecular mechanism underlying this effect. However, we would suggest from our previous observation (Dümmer et al., 2016) and data obtained with BFA on GNOM distribution on the cellular level (Geldner et al., 2005), that the BFA induced disruption of vesicular trans Golgi-traffic has an impact on the distribution of EHB1. We rephrased the particular part within the discussion.

Reviewer #2:

Rath et al. reported the redistribution of a C2 domain-containing protein and negative regulator of light and gravity induced bending, ENHANCED BENDING1 (EHB1), under gravitropic response. The authors carefully designed a new method to visualize cross sections of the root tip and protein expressions fused with GFP under a confocal microscope. They discovered that while EHB1 was symmetrically distributed along the outside layers of the root tip, it redistributed and was preferentially accumulated in the upper side of the root tip away from the gravity direction. They also demonstrated that the asymmetrical pattern was reversible by re-orienting the root vertically. Further, they found that the redistribution was affected by gravitropism inhibitors including protein synthesis inhibitor cycloheximide, fungal toxin Brefeldin A and auxin transport inhibitor NPA, but not cytochalasin D. They also showed the EHB1-GFP signal was dramatically reduced by IAA treatment with and without gravitropic stimulus. The experiments were generally performed in a proper way and the conclusions were reasonable. My concerns include using single transgenic allele and inadequate quantification. Please see my comments below that may help to strengthen the paper.

Major comments:

1. Although both EHB1-GFP and AGD12-GFP are driven by 35S promoter, their distribution patterns are different with and without gravitropic stimulus, with a strong fluorescence signal in the periphery of root tip for EHB1-GFP, and similar pattern less pronounced for AGD12-GFP. I wonder if this effect is due to differentially post-transcriptional regulation between the two proteins, or positional effect where the T-DNA is inserted. If the authors could provide additional alleles of 35S:EHB1-GFP and

35S:AGD12-GFP, and demonstrate the same pattern, it would be more convincing to claim their conclusion. Otherwise, the authors should state the alternative possibility.
At the moment we only investigated on defined line. So indeed, we cannot rule out both possibilities. We added addressed this topic in the discussion.

2. Most of the figures were presented in a qualitative way with the exception of Fig. 7. I wonder if the authors could also provide quantification in other figures, especially in Fig. 5, which showed the effect of different chemicals on the EHB1-GFP signal. The authors showed that IAA dramatically decrease the abundance of the protein. However, comparing Fig. 5A, C, E and G with Fig. 1E, it seems that at least E and G treatment also had an effect on EHB1-GFP intensity. A quantification with multiple seedlings would provide statistical power to the conclusion.

Firstly, we added a quantitative analysis to the new arranged Fig. 4 and Fig. 5 (see above). Regarding the observation that auxin leads to a reduction of EHB1-GFP fluorescence (new Fig. 6), which might explain that the fluorescence decayed at the lower side of the root after a given gravitropic stimulus, we revisited this aspect and repeated this experiment several times. Yet, at the moment the outcome confirmed that auxin abolished the asymmetrically distribution of EHB1-GFP fluorescence, but the overall reduction of EHB1-GFP was quite heterologous in individual seedlings. We decided to spend more effort analyzing the impact of auxin on EHB1 in the near future, since this aspect might be interesting in various ways, in particular in combination with PIN3/7 redistribution, but these experiments would extend the scope of this contribution. At the moment we intent to present the data as an interesting observation and decided to rephrase the corresponding parts in the result and discussion part in a more reluctant way.

3. Most of the figures presented with a black-blue-white-red (low to high) color scale. However, Fig. 1 G-J and Fig. 4 C seem to have only black-white-red colors, as if another color scale was used. If the signal is very strong and no weak blue signal is present, I would expect to see stronger red color and little black signal, which is not the case. Could the authors justify the visual differences in Fig. 1G-J and Fig. 4C?

This could be indeed misleading. Thus, the lookup-table was adjusted to overcome any misinterpretation.

Minor comments:
1. Some figures seem to be mis-referred in the text. For example, in line 147, is Fig. 1B, C supposed to be Fig. 1C, D? In line 152 and 177, should Fig. 1D be Fig. 1B? In line 264, 266 and 269, I think the authors meant Fig. 7 instead of 4.

Corrected

2. Figure 5 labels were also not consistent with the description in the result section.

The images have been rearranged (see comment to reviewer #1).

Finally, we would like to thank both reviewers for their constructive and indeed very helpful comments.