ZLL/AGO10 maintains shoot meristem stem cells during *Arabidopsis* embryogenesis by down-regulating ARF2-mediated auxin response

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Abstract

**Background**: The shoot meristem gives rise to new organs throughout a plant's life by the activity of pluripotent stem cells in the meristem center. Organ initiation at the periphery of the shoot meristem is triggered by the accumulation of the phytohormone auxin at the initiation site. Loss-of-function mutants of the ZWILLE/ARGONAUTE10/PINHEAD (ZLL/AGO10/PNH) gene terminate shoot meristem stem cells late in embryogenesis and can form a leaf or a leaf-like structure instead, indicating that AGO10 activity is required to maintain shoot meristem stem cells undifferentiated.

**Results**: Here, we addressed whether stem cell maintenance by AGO10 involves regulation of auxin. We found that in zll-1 mutants, auxin accumulation and expression of the response reporter DR5:GFP are elevated, and transcription of the Auxin Response Factor 2 (ARF2) gene is upregulated. Downregulation of ARF2 significantly restores stem cells in zll-1 mutants, whereas increased expression of ARF2 enhances differentiation of stem cells in zll-1 mutants. We further found that upregulation of the AGO10 effector gene REVOLUTA restores ARF2 expression and stem cell maintenance in zll-1 embryos.

**Conclusions**: Our results indicate that maintenance of shoot meristem stem cells by AGO10 involves negative regulation of auxin signaling and, via REV-mediated downregulation of ARF2 expression, auxin response.

**Keywords**: ARF2, ARGONAUTE10, Stem cell identity

Background

All above-ground plant organs originate from the shoot apical meristem, which is established during embryogenesis [1, 2]. Within the shoot meristem, the central zone contains three layers of slowly dividing pluripotent stem cells, which respectively give rise to the epidermis, subepidermal cells, and internal tissues. Stem cell daughters enter differentiation pathways in the surrounding peripheral zone, where lateral organs are initiated, and in the underlying rib zone, where the pith of the shoot is formed [3].

Several key regulators of stem cell maintenance have been identified in the past, including a feedback loop between stem cells and underlying niche cells, named the organizing center [4]; regulation of cytokinin synthesis and response [5]; and localized expression of miRNAs [6]. The ARGONAUTE10 (AGO10) gene is required for stem cell maintenance during embryogenesis and for initiation of axillary meristems during postembryonic development. Loss of AGO10 function in the Landsberg erecta (Ler) accession results in stem cells differentiating into a flat apex, leaf-like structures, or normal-appearing leaves [7, 8]. Expression of the stem cell marker CLAVATA 3 (CLV3) is correctly initiated at the transition embryo stage of the zll-1 mutant, but is discontinued at the bent-cotyledon embryo stage [9, 10]. AGO10 encodes a member of the AGO protein family that binds small RNAs and acts as effectors of RNA interference in plants and animals [11, 12]. In contrast to canonical AGO proteins, however, binding of miR165/166 to AGO10 does not result in degradation of the target mRNAs, encoding HDZIP III transcription factors, but in their increase. Therefore, a function of AGO10 as a decoy AGO protein, which limits
loading of miR165/166 onto AGO1 and thus reduces degradation of HDZIP III mRNAs, has been proposed [13].

One of the earliest events in organ initiation by the shoot meristem is the accumulation of auxin via directional cell-to-cell transport [14–16]. Auxin can trigger the degradation of AUX/IAA repressor proteins and consequently the activation of AUXIN RESPONSE FACTORS (ARFs) transcription factors [17, 18]. The Arabidopsis genome contains 23 ARFs [19], specific subsets of which regulate a multitude of developmental processes [20–23]. The stem cell-containing central domain of the meristem appears relatively insensitive to auxin [24] and displays increased mechanical stiffness; these factors have been suggested to limit organ formation [25].

The initiation of differentiated organs in the place of the stem cells in zll mutants raises the question whether AGO10 might negatively regulate auxin function. To address this question, we analyzed the interplay between AGO10 and auxin during embryogenesis. Our results show that AGO10 downregulates both auxin signaling and auxin response, including expression of ARF2. Downregulation of ARF2 levels in zll-1 embryos via an artificial miRNA (amiR) recovers stem cell maintenance. Furthermore, increased REV activity in zll-1 restores ARF2 repression and stem cell maintenance. Together, these results provide a framework of how AGO10 maintains shoot meristem stem cells by negatively regulating auxin signaling.

**Results**

**Auxin signaling and response are increased in zll-1 embryos**
To address whether auxin regulation might be altered in zll-1 mutants, we first monitored auxin response with the pDR5:nlsGFP reporter (Fig. 1, Additional file 1: Figure S1, Additional file 2: Table S1). In wild-type embryogenesis, pDR5:GFP expression was detected from the globular stage onwards at the root pole and additionally from the heart stage onwards in the tips and developing vasculature of the cotyledons. In zll-1 embryos, pDR5:GFP expression levels were increased in all regions where expression was found in wild type. Additional expression was detected in the vasculature of the embryo axis of early heart to torpedo stage embryos and in the epidermis of cotyledonary tips of transition to heart stage embryos. To investigate what causes the increased DR5:GFP response in zll-1, we first monitored expression of the tandem auxin signaling sensor R2D2 [26], where the pRPSSa:DII-n3xVenus (DII-Venus) signal is inversely correlated to auxin signaling and where the pRPSSa:mDII-ntdTomato (mDII-ntdTomato) signal acts as transcription control. In addition, a CLV3xer-CFP transgene was used to monitor shoot meristem stem cells.

In wild-type transition to torpedo stage embryos, expression of the pRPSSa:mDII-ntdTomato reporter was detectable in the cotyledons and at a lower level in the shoot apex and the basal part (Fig. 2). In bent-cotyledon stage embryos, the mDII-ntdTomato signal was close to background level in the majority of the analyzed embryos (Fig. 2 and Additional file 3: Table S2). This indicates that the pRPS5a driver is active in wild-type embryogenesis until the torpedo stage. Expression of the pRPSSa:DII-n3xVenus auxin signaling reporter was detectable in a similar pattern between the transition and the torpedo stages (Fig. 2 and Additional file 3: Table S2). As expected, no signal was detectable in bent-cotyledon stage embryos, where the driver is not active (Fig. 2).

In the zll-1 mutant, the expression of pRPSSa:mDII-ntdTomato did not show any detectable difference compared to wild type (Fig. 2), demonstrating that the pRPS5a driver works as in wild type. In contrast to wild type, however, the DII-Venus signal was not detectable in the majority of zll-1 embryos from transition to torpedo stages (Fig. 2 and Additional file 3: Table S2), indicating increased auxin signaling. We notice that this embryo-wide downregulation of the DII-Venus reporter in zll-1 embryos contrasted the localized upregulation of DR5:GFP expression (Fig. 1). This difference might be due to the reported higher sensitivity of DII-Venus compared to DR5 [27] and the different molecular requirements of the two auxin markers: DII-Venus relies on the presence of the TRANSPORT INHIBITOR RESPONSE1 (TIR1) F-box protein family and on the presence of auxin itself [28–30], and DR5 relies additionally on the presence of ARF transcription factors. One mechanistic interpretation of the different patterns of both reporters is that increased early signaling does not always result in ARF2-dependent transcriptional activities.

**TIR1 and AUXIN SIGNALING F-BOX 1–3 (AFB1–3) auxin receptor mRNAs are targets of miR393 [31, 32], which has been found to preferentially bind to AGO1 [33] and thus could be sensitive to the levels of AGO10. Notably, we found that AFB1 mRNA levels were slightly increased compared to wild type in zll-1 torpedo stage embryos and **TIR1 and **AFB2 mRNA levels in zll-1 bent-cotyledon stage embryos (Fig. 3). Thus, the observed decrease in DII-Venus signal could be partially due to the increase of TIR/AFB expression.

In Arabidopsis, the main source of auxin is from tryptophan via two enzymatic steps, catalyzed by tryptophan amino transferases (TAA1, TARI, and TAR2) [34, 35] and the YUCCA (YUC) family monoxygenases [36]. We found that the expression of **YUC1 and **YUC4 was weakly upregulated in the zll-1 mutant compared to wild type (Fig. 3), whereas **TAA1 and **TAR2 expression was not significantly changed (Additional file 4: Figure S2).

In summary, these results indicate that between transition and torpedo stages, zll-1 embryos display...
higher levels of auxin synthesis and signaling compared to wild type.

**ARF2 levels in zll-1 mediate stem cell termination**

We next asked whether increased auxin signaling might contribute to the shoot meristem defects in zll-1 mutants through ARF auxin effector genes. For an initial characterization we chose ARF2 as a TAS3-regulated ARF gene [37], and ARF6 as a miR167-regulated ARF gene [38], which are expressed during embryogenesis but not in the embryonic shoot apex [21]. Because no loss-of-function mutants were available in the Ler ecotype, we downregulated ARF2 and ARF6 mRNA levels via artificial miRNAs (amiR). Changing ARF6 expression had no effect on shoot meristem formation in wild type or in zll-1 (Additional files 5, 6, 7, and 8: Tables S3–S6) and was not further pursued.

Expression of p35SamiR-ARF2 strongly reduced ARF2 mRNA levels detected by semi-quantitative reverse transcriptase polymerase chain reaction (sqRT-PCR) in torpedo stage embryos (Fig. 4a), but did not have any effect on the shoot meristem development in wild type (not shown), in accordance with previous findings [23]. By contrast, in zll-1 bent-cotyledon stage embryos, p35SamiR-ARF2 expression partially rescued stem cell maintenance as measured by the expression of the stem cell marker pCLV3:GFP-er ($p < 0.001$; Fig. 4b,c) and the presence of an active shoot meristem at the seedling stage (Additional file 9: Table S7).
In the converse experiment, we upregulated ARF2 mRNA levels by expressing a TAS3-resistant pARF2:ARF2m transgene in order to avoid degradation of ARF2 mRNA via the TAS3 siRNA. In the zll-1 background, this resulted in a significant (p < 0.001) reduction of pCLV3:GFP-er expression in bent-cotyledon stage and mature embryos (Fig. 5a) and of shoot meristem maintenance (Fig. 5b, Additional file 10: Table S8). In addition, the severity of shoot meristem defects was enhanced (Fig. 5b, p < 0.001). By contrast, pARF2:ARF2m expression did not cause any obvious phenotypic change in the shoot apical meristem when expressed in the wild type (Fig. 5b). This suggests that ARF2 levels are not a limiting factor in shoot meristem maintenance in wild type, but are in zll-1, consistent with the increased auxin accumulation in zll-1.
Together these results suggest that downregulation of ARF2 expression is required for shoot meristem stem cell maintenance during embryogenesis by AGO10 activity.

REVOLUTA mediates downregulation of ARF2 expression by AGO10

One possible reason why reduction of ARF2 activity suppresses shoot meristem termination in zll-1 embryos is that AGO10 negatively regulates ARF2 expression levels. Indeed, we found by qRT-PCR that ARF2 mRNA levels were 3–4-fold increased in zll-1 torpedo stage embryos compared to wild type, whereas ARF3 and ARF4 levels were not affected (p < 0.001; Fig. 6a, Additional file 11: Figure S3).

Next we analyzed expression of the transcriptional reporter pARF2:ntd-Tomato (pARF2:ntdTomato) during embryogenesis. In wild type, pARF2:ntdTomato was hardly detectable from heart to torpedo stages and was detected throughout the embryo at the bent-cotyledon stage (Fig. 6b, Additional file 12: Figure S4). By contrast, in zll-1 heart to bent-cotyledon stage embryos, pARF2:ntdTomato was uniformly expressed at higher levels compared to wild type (Fig. 6b, Additional file 12: Figure S4). We note that this expression pattern differs from the one reported in the Columbia accession [21], which could be due to different ARF2 expression levels in the Ler accession used in our experiments.

In summary, these data indicate that AGO10 is required to downregulate ARF2 transcription from heart to torpedo stages throughout the embryo.

AGO10 prevents accumulation of miR165/166 in the embryonic shoot meristem and thus promotes expression of their target HD-ZIP III genes [13]. We therefore asked whether HD-ZIP III activity might mediate repression of ARF2 transcription via AGO10. To this end we introgressed the miR-resistant rev-10d mutation [39], which as
Fig. 4  (See legend on next page.)

A

B

C

| Transition to Heart | Heart | Torpedo | Bent-Cotyledon | Mature |
|---------------------|-------|---------|---------------|--------|
| **pCLV3:GFP-er**    |       |         |               |        |
| n=100               | n=47  | n=58    | n=210         |        |
| **pCLV3:GFP-er**    |       |         |               |        |
| n=100               | n=220 | n=164   | n=74          |        |
| **pCLV3:GFP-er**    |       |         |               |        |
| n=74                | n=64  | n=64    |               |        |
| **pCLV3:GFP-er**    |       |         |               |        |
| n=64                | n=64  | n=64    |               |        |

Legend:
- no signal
- weak
- strong

1: Ler
2: zll-1
3: zll-1amiR-arf2
a single mutant does not display any shoot meristem defect (not shown), into the zll-1 mutant. This resulted in a significant rescue of shoot meristem formation in zll-1 embryos ($p < 0.001$; Additional file 13: Figure S5A), including the expression of the stem cell marker pCLV3:GFP-er ($p < 0.001$; Fig. 4b, Additional file 13: Figure S5B).

qRT-PCR studies demonstrated that the rev-10d mutation largely suppressed the upregulation of ARF2 mRNA levels in the zll-1 mutant (Fig. 6a, $p < 0.001$), and that inducible p35S:GR-REVd activity repressed ARF2 expression (Fig. 6c, $p < 0.01$). Addition of cycloheximide abolished the effect of REV on ARF2 mRNA (Fig. 6c),
indicating that it is indirect. Likewise, rev-10d suppressed the expression levels of the ARF2 transcriptional reporter in zll-1 embryos (Additional file 12: Figure S4).

Taken together, this indicates that AGO10 represses ARF2 transcription in the embryonic shoot meristem through REV activity.

**Discussion**

During embryonic shoot meristem formation, the two basic functions of the meristem need to be established in spatially separated domains: stem cell maintenance in the meristem center, and organ initiation in the periphery [3, 40]. Several mutants of shoot meristem development result in an inactive stem cell pool, resulting in a terminated apex without the initiation of an organ [41–43]. By contrast, zll mutant embryos frequently form a leaf structure in place of the stem cell pool in the meristem center [8, 44]. This defect first becomes visible at the bent-cotyledon embryo stage when zll-1 embryos are unable to maintain expression of the shoot meristem stem cell marker CLV3 [10]. In this study, we have addressed the underlying mechanisms of this phenotype and analyzed the role of auxin and AGO10 interaction in stem cell maintenance.

**AGO10 negatively regulates auxin signaling and response during embryogenesis**

Our results provide several lines of evidence that increased auxin function in zll-1 contributes to the failure to maintain shoot meristem stem cells in the embryo, as detected by the loss of CLV3 expression and the ectopic organ formation in place of the stem cells. First, the perception capacities and auxin levels were elevated in the zll-1 mutant throughout the embryo. Second, reduction
of ARF2 levels partially suppressed stem cell termination in zll-1, whereas increased ARF2 levels increased stem cell defects.

In protoplast transfection assays, ARF2 acts as transcriptional repressor of DR5:GFP [45], which would suggest that upregulation of DR5:GFP in zll-1 is not a direct response of elevated ARF2 levels and therefore other factors are probably involved. We consistently found that, in addition to ARF2, other components of auxin response, such as TIR1/AFB and YUC genes, were upregulated in zll-1 mutants, albeit at weaker levels.

How is the upregulation of ARF2 accomplished? Previous studies showed thatAGO10 promotes expression of the transcription factor REV by blocking degradation of its mRNA via miR165/166 [13]. Our results show that, in turn, REV mediates downregulation of ARF2 by AGO10, because increased REV levels in the zll-1 mutant reduced ARF2 expression and suppressed stem cell defects in the zll-1 mutant. It is likely that ARF2 repression by REV is not direct, because repression did not take place when protein synthesis was blocked. Consistent with this notion, REV was not found to bind to ARF2 in a genome-wide map of REV binding sites [46].

Curiously, a previous study showed that overexpression of REV in seedlings directly activates expression of auxin biosynthesis genes of the tryptophan-dependent indole-3-pyruvic acid biosynthetic pathway, TAA1 and YUC5 [46]. In our studies, however, we found that YUC1 and YUC4 levels and possibly auxin accumulation were upregulated in zll-1 embryos, suggesting that AGO10 affects auxin synthesis by a mechanism independent of REV, or that REV acts differentially on auxin synthesis during embryo and seedling development.

These findings indicate that AGO10 is required to reduce auxin activity in the embryo and that this process is essential for maintaining shoot meristem stem cells [14, 47].

Genetic interaction studies and expression experiments indicate that AGO10 is required to potentiate WUS-mediated maintenance of CLV3 expression in the embryonic shoot meristem stem cells [10]. In contrast to WUS, which is confined to the developing shoot meristem region, AGO10 is expressed in a broader pattern, including the shoot meristem and the pro-vascularure [10]. However, localized expression of AGO10 in the vasculature is sufficient for shoot meristem development, suggesting that AGO10 can act non-cell autonomously. Consistent with the broad AGO10 expression pattern in embryos, we found enhanced auxin signaling and response and increased ARF expression throughout the zll-1 embryo. Because organ initiation in the shoot meristem requires the accumulation of auxin at the initiation site through directional transport from surrounding tissues [48], one interesting question to be answered in the future is whether stem cell loss in zll-1 embryos is the consequence of enhanced auxin activity inside or outside the shoot meristem.

Conclusion
In this study, we show that the ARGONAUTE family member AGO10 negatively regulates auxin signaling during embryogenesis. Consequently, the conversion of stem cells into an ectopic leaf-like organ in the zll-1 mutant appears to be due to upregulated auxin activity involving increased transcription of the auxin response factor ARF2.

Methods
Arabidopsis plants were grown as described previously [10]. The shoot apical meristem of Ler, zll-1, and transgenic lines was analyzed 14 days after germination. Plant transformation was done by the Agrobacterium-mediated floral dipping method [49]. Wild-type and 35S:GR-REVd seedlings were grown on Murashige and Skoog medium plates in long-day conditions at 22 °C for 4 days. Induction of GR-REVd was done as described previously [50].

Constructs
Primers for three artificial miRNAs of ARF2 were designed using the WDM3 online software and amiRNAs were amplified as described previously [51]. Specificity of amiR sequences for ARF2 was confirmed using BlastN. The artificial miRNAs were subcloned into the BamHI site of the pEG287 vector. The amiR-ARF2 with a 35S promoter upstream and a Nos-Terminator downstream were cloned in HindIII/EcoRI sites of a pGreenII binary vector. The promoter of ARF2 was amplified using forward primer 5′-ACTAAGCTTACCTTACCCAGGA-3′ and reverse primer 5′-ACTAAGCTTACCTTACCCAGGA-3′, and cloned into the HindIII restriction site of a modified pGreenII vector containing a ligation-independent cloning (LIC) tail [52]. The ARF2 coding region was amplified using forward primer 5′-TAGTTGGAATAGGATTTCGTTGGATCCTTAAGAGTTC-3′ and reverse primer 5′-ACTAAGCTTACCTTACCCAGGA-3′, and cloned into the HindIII restriction site of a modified pGreenII vector containing a ligation-independent cloning (LIC) tail [52]. The ARF2 coding region was amplified using forward primer 5′-ACTAAGCTTACCTTACCCAGGA-3′ and reverse primer 5′-ACTAAGCTTACCTTACCCAGGA-3′, and cloned into the HindIII restriction site of a modified pGreenII vector containing a ligation-independent cloning (LIC) tail [52].
The ARF2 transcription marker sequence was amplified with forward primer 5′-TAGTTGGAATAGATTCTCG TCCTTTAGGCTTCGGTTTCTC-3′ and reverse primer 5′-AGATATGAGTTTCGTTGGA TTCTAAAGGTTCCCGAGCCTG-3′, and subcloned into a modified pGreenII vector containing a LIC tail containing the ARF2 promoter.

**Microscopy**

Embryos were dissected from maternal tissues and mounted in 10% glycerol. They were analyzed with a Zeiss AxioImager. A1 microscope (Carl Zeiss Microscopy, Jena, Germany) or a Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). The images were processed using Adobe Photoshop Elements 2.0.

The integrated signal intensity of each embryo was measured in a median plane and calculated using the ROI tool of Image J software. Analysis of the measured data and drawing of bar plots were done using Microsoft Excel software.

**Semi-quantitative PCR of ARF2, ARF3, and ARF4**

To measure the efficiency of amiR-ARF2, RNA of ztl-1 and ztl-amiR-ARF2 lines was extracted using a Qiagen RNeasy kit (Qiagen, Hilden, Germany). cDNA amplification was done with the Qiagen cDNA synthesis kit. Amplification of cDNA sequences were performed using forward primer 5′-TTCCGATGCTTTACCAGAAGGT-3′ and reverse primer 5′-TTGAGTCTGTCCTCCATTCATGGT-3′ for ARF2; forward primer 5′-GATTCCAGAGGTCTTGCAGGTC AAGAAATTTTCC-3′ and reverse primer 5′-CAAC GCAGGGGACGCGCTC-3′ for ARF3; and forward primer 5′-TTCCTCGGTTCTTCCACACTC-3′ and reverse primer 5′-AGGAAATTTTCCACCTGCCAAGAC CCTTGGAAACC-3′ for ARF4. As reference, ACT7 (At5g99810) was amplified with forward primer 5′-GG TTAGATATTCAGCCACTTGCTG-3′ and reverse primer 5′-TGTGAGATCCGACCCAGGACTC-3′ [53].

**Quantitative PCR**

Torpedo stage embryos were dissected out of the ovule, and RNA was extracted using the Qiagen RNeasy kit. cDNA was generated using the Invitrogen SuperScript III first-strand synthesis system for qPCR (cat. no. 11752–050; Life Technologies, Carlsbad, U.S.A.).

qRT-PCRs were done using the Roche Lightcycler 480 SYBR Green I kit (Cat. No. 04707516001; Roche Diagnostics GmbH, Mannheim, Germany), with forward primer 5′-TGCTGTCCGCTTGGTCAGG-3′ and reverse primer 5′-GGCCGCTTGGTCGTTCCAG-3′ of ARF2; forward primer 5′-CAAGGAGGGTCTGAGGCTGCA-3′ and reverse primer 5′-CAAGGAGGGGGAAGCAGCC GTC-3′ of ARF3; and reverse primer 5′-TCTCCAGACTC-3′ and reverse primer 5′-ATGGGGTT TCCGGGTTGGGT-3′ of ARF4; forward primer 5′- CGAATATAAGCCATATTACGC-3′ and reverse primer 5′-CCATTACAGAAGAGAGTCC-3′ for TAA1; forward primer 5′-GCTCTTCATCCTGTT CAAGAGCAGC-3′ and reverse primer 5′-CTCTGTC TTACAAAAGCCCATC-3′ for TAR2; forwards primer 5′-GAGAGACGCAAATCAGG-3′ and reverse primer 5′-GAGGTAAAGACAAAACGAGAC CGTG-3′ for YUC1; and forward primer 5′-ATGGGG CACTTGTGAGAGATC-3′ and reverse primer 5′- CGGACCAAGACGAAGATC-3′ for YUC4. For TIR1, we used forwards primer 5′-TGAGGAAGACTAGATAAG GGAAGTCG-3′ and reverse primer 5′-CACCAAGAA ACAGACAGATC-3′ for AFB1; forwards primer 5′-ACTTGGTCTGCCGCTGTAGAG-3′ and reverse primer 5′-CTCTGGAGGTTCCTTACATGACTTC-3′ for AFB2; forwards primer 5′-CAAGTATGAAACATGCG ATCCCTGTTG-3′ and reverse primer 5′-CTCTTGCCAT CGGTTATATTCCATCTCG-3′ for AFB3; and forwards primer 5′-AAGGATCTGCTCTATGTGTTGAATGGATG-3′ and reverse primer 5′-AACCTCTTCTCTCTCAT CTCTTCTATTG-3′. At4g26410 [54] was used as a reference gene, and amplified with forward primer 5′-GAGGTAAGTGCTTCTCCATGAC-3′ and reverse primer 5′-GGTCCGACTC-3′.

**Additional files**

Additional file 1: Figure S1. DRS::GFP signal is upregulated in ztl-1 embryos. Integrated fluorescence intensity of DRS::GFP signal of whole embryos at the indicated stages. n numbers of embryos analyzed, n.s. not significant. ***p < 0.001. (PPT 107 kb)

Additional file 2: Table S1. Increased auxin response in the ztl-1 mutant. (DOC 51 kb)

Additional file 3: Table S2. Auxin-Venus levels are reduced in ztl-1 and reverse mutant. (DOC 65 kb)

Additional file 4: Figure S2. Transcript levels of auxin biosynthesis genes TAA1 and TAR2. TAA1 (A) and TAR2 (B) transcript levels are not significantly altered between ztl-1 and Ler wild-type embryos. qRT-PCR transcript levels relative to reference gene At4g26410 and standard deviation of three biological replicates are shown. Student’s t-test was used to calculate p-values. (PPT 112 kb)

Additional file 5: Table S3. Increased ARF6 expression does not affect shoot apical meristem development in ztl-1. (DOC 43 kb)

Additional file 6: Table S4. Increased ARF6 expression does not affect shoot apical meristem development of wild type. (DOC 43 kb)

Additional file 7: Table S5. Reduction of ARF6 expression does not affect ztl-1 shoot apical meristem development. (DOC 52 kb)

Additional file 8: Table S6. Reduced ARF6 expression does not affect shoot apical meristem development in wild-type background. (DOC 53 kb)

Additional file 9: Table S7. Reduced ARF2 expression partially suppresses ztl-1 shoot apical meristem defects. (DOC 42 kb)

Additional file 10: Table S8. Increased ARF2 expression enhances the frequency of shoot meristem termination in ztl-1. (DOC 48 kb)

Additional file 11: Figure S3. Expression of ARF2 but not ARF3 and ARF4 is negatively regulated by AGO10 and REV. ARF mRNA levels in torpedo stage embryos of the indicated genotypes relative to wild type.
At4g26410 transcript levels are normalized to the reference gene At4g26410. Significance tested by Student’s t-test is indicated. **p < 0.01, ***p < 0.001. All other comparisons did not show a significant difference. (PPT 123 kb)

Additional file 12: Figure S4. Upregulation of pARF2:ntTomato expression in zll-1 embryo is suppressed by rev-10d. Percentages of pARF2:ntTomato signal strength in zll-1 embryos of the indicated genotypes are shown. Chi-square test was used to calculate p-values. **p < 0.01, ***p < 0.001. n number of analyzed embryos. (PPT 120 kb)

Additional file 13: Figure S5. Shoot meristem development is partially restored in rev-10d by rev-10d. (A) The severity of shoot meristem defects in zll-1 is alleviated by the gain-of-function mutation rev-10d. (B) Decreased pCUC2:GFP expression levels in zll-1 bent-cotyledon and mature embryos are partially suppressed by rev-10d. Genotypes and embryo stages are indicated. Chi-square test was used to calculate p-values. **p < 0.01, ***p < 0.001. n number of analyzed embryos. Shoot meristem defects: empty apex differentiated stem structure, no organ formation; pin single filamentous structure; 1 L one central leaf, 2 L termination into two leaves; wt wild-type-like. (PPT 162 kb)

Abbreviations
ARF1–3: AUXIN SIGNALING F-BOX1 auxin receptor 1–3; AGO10: ARGONAUTE 10; amiRNA: artificial miRNA; ARF2: AUXIN RESPONSE FACTOR 2; ATP-2: mutated ARF2; CLS: CLAVATA 3; GFP: green fluorescent protein; LR: Landsberg erecta; qRT-PCR: quantitative reverse transcriptase polymerase chain reaction; REV: REVOLUTA; TAA1: TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1; TARI1: TRYPTOPHAN AMINOTRANSFERASE RELATED 1/2; TIR1: TRANSPORT INHIBITOR RESPONSE1; YUC1–4: YUCCA 1–4; ZLL: ZWILLE.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
FR performed all experiments, ET prepared the amiRNA constructs and TL supervised the project. All authors read and approved the final manuscript.

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