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Vascular signalling mediated by ZWILLE potentiates WUSCHEL function during shoot meristem stem cell development in the *Arabidopsis* embryo

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Stem cells are maintained in an undifferentiated state by signals from their microenvironment, the stem cell niche. Despite its central role for organogenesis throughout the plant's life, little is known about how niche development is regulated in the *Arabidopsis* embryo. Here we show that, in the absence of functional ZWILLE (ZLL), which is a member of the ARGONAUTE (AGO) family, stem cell-specific expression of the signal peptide gene *CLAVATA3* (*CLV3*) is not maintained despite increased levels of the homeodomain transcription factor WUSCHEL (WUS), which is expressed in the organising centre (OC) of the niche and normally promotes stem cell identity. Tissue-specific expression indicates that *ZLL* acts to maintain the stem cells from the neighbouring vascular primordium, providing direct evidence for a non-cell-autonomous mechanism. Furthermore, mutant and marker gene analyses suggest that during shoot meristem formation, *ZLL* functions in a similar manner but in a sequential order with its close homologue *AGO1*, which mediates RNA interference. Thus, *WUS*-dependent OC signalling to the stem cells is promoted by *AGO1* and subsequently maintained by a provascular *ZLL*-dependent signalling pathway.

KEY WORDS: *Arabidopsis* embryogenesis, Meristem, Stem cell niche, *ZWILLE* (*PINHEAD*; *AGO10*)

INTRODUCTION

Stem cells are located in specialised microenvironments, stem cell niches, where signals from the neighbouring cells maintain them in a pluripotent state. This principle was recognised several decades ago in animals and plants (Schofield, 1978; Stewart and Dermen, 1970), but only recently have the regulatory mechanisms started to be unravelled.

In the wild-type *Arabidopsis* shoot meristem, three layers of stem cells are located at the very tip and give rise to all shoot organs formed in a plant's life. They are maintained in an undifferentiated state by signals that depend upon expression of the homeodomain transcription factor WUSCHEL (WUS) in a small underlying cell group termed the organising centre (OC) (Mayer et al., 1998). The stem cells in turn express CLAVATA3 (CLV3), a signal peptide that acts to restrict *WUS* transcription via the CLV1/CLV2 receptor kinase signalling cascade (Brand et al., 2000; Schoof et al., 2000). This feedback loop between the OC and stem cells provides a mechanism to control the size of the stem cell pool. Additional pathways have been identified that work in parallel to the WUS/CLV3 loop in regulating stem cell identity (Brand et al., 2002; Lenhard et al., 2002; McConnell et al., 2001; Prigge et al., 2005; Vroemen et al., 2003). Several studies suggest that during postembryonic development, tissues surrounding the meristem also provide important information for shoot meristem maintenance (reviewed by Tucker and Laux, 2007), including the internal (L3) cell layers (Stuurman et al., 2002; Szymkowiak and Sussex, 1992) and the adaxial sides of leaf primordia (McConnell et al., 2001; Waites et al., 1998). Furthermore, the sites of shoot meristem regeneration in tissue culture (Brossard, 1979; Projetti and Chriqui, 1986), in sunflower hybrids (Chiappetta et al., 2006), or after *KNOTTED-LIKE HOMEOBOX* (*KNOX*; also known as *KNATM* – TAIR) overexpression (Chuck et al., 1996; Nishimura et al., 2000), correlate with the position of vascular cells. However, the underlying mechanism remains elusive.

Little is known about how the shoot meristem stem cells are formed in the embryo. After separation of protoderm and inner cells, the onset of *WUS* expression in four sub-epidermal apical cells of the 16-cell embryo, which after several asymmetric cell divisions give rise to the OC (Laux et al., 2004), is the first indication of stem cell niche development during *Arabidopsis* embryogenesis. At the same stage, the cells below the OC precursor cells start to elongate and form the vascular primordium (Jürgens and Mayer, 1994; Mansfield and Briarty, 1991). The shoot meristem stem cells, however, cannot be distinguished before the middle stages of embryogenesis, when expression of *CLV3* is detected (Fletcher et al., 1999).

Mutagenesis screens identified the ARGONAUTE (AGO) family member *ZWILLE* (*ZLL*; also known as *PINHEAD* and *AGO10*) as a factor involved in shoot meristem development (Lynn et al., 1999; Moussian et al., 1998). AGO proteins have been revealed as central components of RNA-induced silencing complexes (RISC) in animals and plants, where they bind small RNA molecules to target messenger RNAs for degradation, translational inhibition, or genomic DNA for methylation (reviewed by Peters and Meister, 2007; Vazquez, 2006). *zll* mutant embryos form terminally differentiated cells and organs instead of shoot meristem stem cells (Lynn et al., 1999; Moussian et al., 1998), but the mechanism underlying the function of the *ZLL* gene has remained unclear. Notably, meristems formed postembryonically can give rise to fertile plants with indeterminate inflorescences, indicating that *ZLL* is specifically required for embryonic shoot meristem development.

In this study we provide direct evidence that during embryonic patterning, *ZLL* acts from the emerging vascular primordium to mediate *WUS* function and maintain shoot meristem stem cells in an undifferentiated state, thereby indicating the presence of a novel signalling pathway downstream of ZLL.

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MATERIALS AND METHODS

Plant work

Plants were grown as described previously (Laux et al., 1996). The *zll-1*, *zll-15* (Moussian et al., 1998) and *ago1-8* (Newman et al., 2002) mutants are in the Landsberg *erecta* (L*er*) background and *ago1-1* (Bohmert et al., 1998) is in the Columbia background. The respective wild-type plants were used as controls. Transgenic *ago1-1* lines were backcrossed to L*er* and showed the same effect as the original Columbia lines.

Transgenes

The *pCLV3:CFP-ER* transgene was generated by replacing the *GFP* reading frame of *pCLV3:GFP-ER* (Lenhard and Laux, 2003) with *CFP*. For the *pZLL:YFP-ZLL* gene, a modified *YFP* sequence lacking a stop codon was inserted immediately prior to, and in frame with, the *ZLL* coding sequence in the context of an 8 kb genomic fragment. The *ZLL* promoter was then replaced with the *ARR5*, *AS1*, *AS2* and *ATHB8* promoters (sequences of primers used to amplify these promoters are available upon request) to generate tissue-specific expression constructs. The *gWUS-GFP$_3$* transgene contains a 3x*GFP* gene inserted immediately prior to the stop codon of a 15 kb *Bam*HI genomic fragment of *WUS*. For ectopic *WUS* expression with the pOpL two-component system (Moore et al., 1998), the *ATRPS5A* promoter (Weijers et al., 2001) was fused to the synthetic transcription factor gene *LhG4*, transformed into plants as the driver line, and crossed to an effector line carrying the *pOp:WUS*(69.3) construct (Schoof et al., 2000). F1 progeny carrying both transgenes were germinated on 1/2 MS media and scored for phenotype at 4 and 11 days post-germination. All plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 [pMP90 (Koncz and Schell, 1986)] and transformed into plants using the floral dip method (Clough and Bent, 1998).

Microscopy and image analysis

Embryos were dissected from ovules on slides using fine-tip syringes in 10% glycerol and viewed on an Axioscope fluorescence microscope (Zeiss). Embryos were stained with DAPI (1 mg/mL) for 5 minutes and mounted in 50% glycerol in 1×PBS. Images were captured using Axiovision 4.4 software (Zeiss) and figures were generated using Photoshop 7.0 (Adobe).

RESULTS AND DISCUSSION

Histological sections of *zll* embryos revealed no abnormalities in stem cell development until late stages of embryogenesis, when the cells at the stem cell position, unlike the small pluripotent stem cells in the wild type, enlarge and become vacuolated, indicating their differentiation. This defect is incompletely penetrant in all known *zll* alleles, and even in the strongest alleles only 90% of the seedlings lack functional stem cells, whereas the remaining seedlings are indistinguishable from wild type. To study how *ZLL* affects stem cell development in the embryo, we analysed expression of the shoot meristem stem cell marker *pCLV3:GFP-ER* (Lenhard and Laux, 2003). No difference was observed in the *pCLV3:GFP-ER* expression pattern between *zll-1* and wild-type embryos from the earliest detection at transition stage until the early torpedo stage (Fig. 1, compare A,B with D,E; see Table S1 in the supplementary material), suggesting that *ZLL* does not have a discernable role in the establishment of *CLV3* expression. However, from torpedo stage onwards, when *pCLV3:GFP-ER* extended into the three layers of stem cells in wild type (Fig. 1B,C), in the majority of *zll-1* embryos the intensity and number of cells expressing *pCLV3:GFP-ER* gradually decreased (Fig. 1E,F; see Table S1 in the supplementary material). The frequency of embryos showing strongly reduced or no *pCLV3:GFP-ER* expression correlates closely with the frequency of *zll-1* seedlings lacking the shoot meristem (compare Table S1 with Table S6 in the supplementary material). This suggests that ZLL activity is required to maintain stem cells of the embryonic shoot meristem, but not to initiate them.

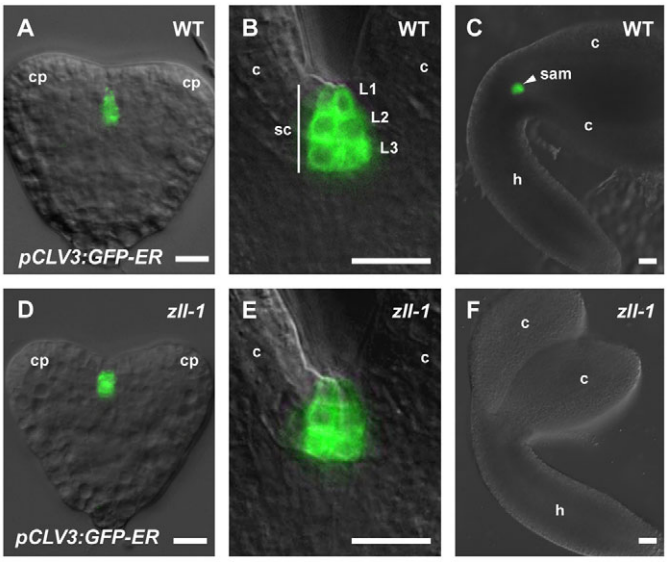

Fig. 1. *pCLV3:GFP-ER* expression is altered in *zll-1* embryos. (**A-C**) *pCLV3:GFP-ER* expression (green) in wild-type (WT) *Arabidopsis* embryos. (A) Transition stage embryo (B) Close-up of stem cells in a torpedo stage embryo. (C) Bent cotyledon stage embryo. (**D-F**) *pCLV3:GFP-ER* expression in *zll-1*. (D) Transition stage embryo. (E) Close-up of stem cells at torpedo stage showing slightly weaker expression than in wild type. (F) Bent cotyledon stage embryo showing no *pCLV3:GFP-ER* expression. cp, cotyledon primordium; c, cotyledon; sc, stem cells; L1, L2 and L3, tissue layers 1, 2 and 3, respectively; sam, shoot apical meristem; h, hypocotyl. Scale bars: 10 μm in A,B,D,E; 20 μm in C,F.

In previous studies, *ZLL* mRNA was detected throughout the embryo at early globular stage and subsequently became restricted to the vasculature and, at lower levels, to the shoot meristem and the adaxial sides of the cotyledons (Lynn et al., 1999; Moussian et al., 1998). By contrast, using anti-ZLL antibodies, ZLL protein was only detectable in the vascular primordium of whole-mount embryos (Moussian et al., 2003). To clarify this discrepancy, we constructed an YFP-ZLL fusion protein that rescued the *zll-1* mutant phenotype (see Table S2 in the supplementary material) when expressed from the *ZLL* promoter. YFP-ZLL fusion protein was first detected in all cells of the proembryo between the 2-cell (Fig. 2A) and 8-cell stages. During subsequent stages, YFP-ZLL became restricted to the emerging vascular primordium (Fig. 2B) and the adaxial domain of the cotyledons (Fig. 2C,D), but was barely detectable in the shoot meristem until maturity, when expression there increased (see Fig. S1 in the supplementary material). Thus, YFP-ZLL localisation closely mimicked the *ZLL* mRNA expression pattern, indicating that all cells where mRNA is detected also produce ZLL protein. It is plausible that the failure to detect ZLL protein in the shoot apex and the adaxial cotyledons in previous studies was due to the lower sensitivity of whole-mount immunodetection in this experiment.

Based on this dynamic expression pattern, the shoot meristem, (Moussian et al., 1998), the adaxial side of the cotyledons (Lynn et al., 1999; Newman et al., 2002), and the vasculature (Moussian et al., 2003) have been discussed as potential sites of ZLL function in embryonic stem cell development. To clarify this, we first compared *pZLL:YFP-ZLL* expression with that of the stem cell niche markers *gWUS-GFP$_3$* and *pCLV3:CFP-ER* during all relevant stages of embryogenesis. At the early globular stage, the *gWUS-GFP$_3$*-expressing OC precursor cells were completely encompassed by strong YFP-ZLL expression (Fig. 2B). However, from the transition

DEVELOPMENT

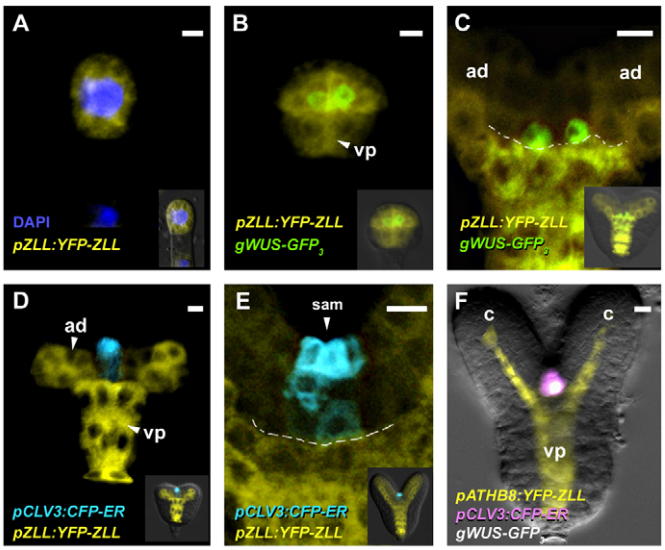

Fig. 2. *pZLL:YFP-ZLL* expression relative to the stem cell niche. (**A**) YFP-ZLL (yellow) accumulates in the apical cell of the 2-cell stage *Arabidopsis* embryo (blue, DAPI). (**B**,**C**) YFP-ZLL localisation relative to WUS-GFP$_3$ (green). (B) 16- to 32-cell stage embryo. (C) Early heart stage embryo. The dashed line indicates the uppermost layer of YFP-ZLL-expressing cells. (**D**,**E**) YFP-ZLL localisation relative to *pCLV3:CFP-ER* expression (cyan). (D) Early heart stage embryo. (E) Late heart stage embryo. The dashed line in E indicates the same boundary as in C. (**F**) Pro-vascular *pATHB8:YFP-ZLL* (yellow) expression does not overlap with *pCLV3:CFP-ER* (pink) or *gWUS-GFP$_3$* (white) expression during embryogenesis. A torpedo stage embryo is shown. Insets show the differential contrast/fluorescent overlay. c, cotyledon; sam, shoot apical meristem; vp, vascular primordium; ad, adaxial cotyledon domain. Scale bars: 5 μm.

stage until late torpedo, when abnormal differentiation of stem cells in *zll-1* embryos is observed, YFP-ZLL was barely detectable in either the OC or stem cells (Fig. 2C-E). To determine where *ZLL* is necessary for meristem development, the YFP-ZLL fusion protein was expressed using different promoter sequences at specific embryo stages and in specific embryonic regions overlapping with the endogenous *ZLL* expression pattern (Fig. 3). The expression pattern of each transgene was verified by localisation of the YFP signal, and function was assayed by rescue of shoot meristem stem cells in the *zll-1* mutant (Fig. 3; see Table S2 in the supplementary material). Notably, *ZLL* expression in the stem cells via the *CLV3* promoter (Fig. 3D-F) and in adaxial tissues of the cotyledons via the *ASYMMETRIC LEAVES 2* (*AS2*) (Iwakawa et al., 2002) promoter (Fig. 3G-I) was unable to rescue stem cell development in *zll-1* embryos. By contrast, expression of *YFP-ZLL* restricted to the vascular primordium (Fig. 3J-L) from the *Arabidopsis HOMEOBOX GENE 8* (*ATHB8*) (Baima et al., 1995) promoter did rescue stem cell maintenance. Importantly, co-expression with the *gWUS-GFP$_3$* or *pCLV3:CFP-ER* reporter genes demonstrated that *pATHB8:YFP-ZLL* expression does not overlap at any stage in embryo development with the cells that give rise to the shoot meristem stem cell niche (Fig. 2F). Thus, ZLL function in the vascular primordium appears to be sufficient for stem cell niche development, indicating a non-cell-autonomous function, whereas its expression in the stem cells and adaxial sides of the cotyledons is neither required nor sufficient for stem cell maintenance. Notably, expression of *YFP-ZLL* in the vascular primordium of the embryo axis from the *ARABIDOPSIS RESPONSE REGULATOR 5* (*ARR5*)

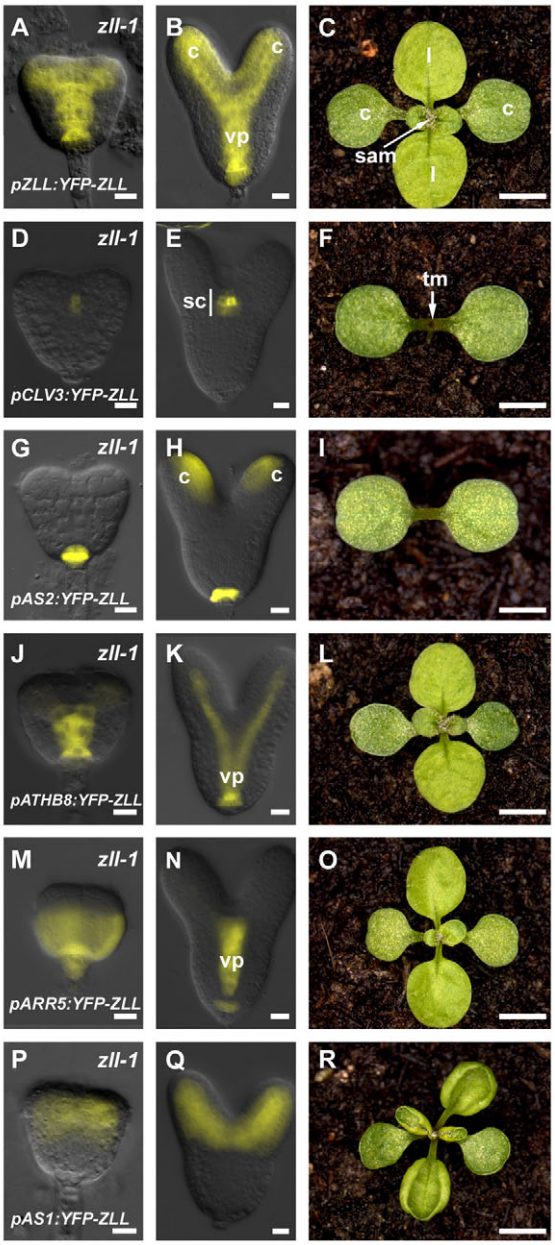

Fig. 3. *ZLL* functions from outside of the stem cells during embryogenesis. Expression of (**A-C**) *pZLL:YFP-ZLL*, (**D-F**) *pCLV3:YFP-ZLL*, (**G-I**) *pAS2:YFP-ZLL*, (**J-L**) *pATHB8:YFP-ZLL*, (**M-O**) *pARR5:YFP-ZLL* and (**P-R**) *pAS1:YFP-ZLL* in *zll-1* mutants. Transition/early heart and torpedo stage *Arabidopsis* embryos are shown, and seedlings are at 16 days post-germination. c, cotyledon; sam, shoot apical meristem; vp, vascular primordium; l, leaf; sc, stem cells; tm, terminated meristem. Scale bars: 10 μm, except 2.5 mm in C,F,I,L,O,R.

(D'Agostino et al., 2000) promoter (Fig. 3M-O), or in the apical parts of the embryo (including the vascular primordium of the cotyledons) via the *ASYMMETRIC LEAVES 1* (*AS1*) (Byrne et al., 2000) promoter (Fig. 3P-R), led to a complete rescue of the *zll-1* phenotype. Since both expression domains overlap only in the provascular cells that lie immediately underneath the stem cell niche, these cells might have a specific role in stem cell maintenance.

Provascular *ZLL* function could affect stem cell development independently of *WUS*, or could interact with *WUS* signalling from the OC. Contrary to the former possibility, ectopic expression of *ZLL*

in embryos from the strong *AGO1* promoter was unable to alter *CLV3* mRNA or *pCLV3:GFP-ER* expression patterns (see Fig. S2 in the supplementary material), despite the potential of most apical and some central embryo cells to activate *CLV3* expression ectopically (E.J.T. and T.L., unpublished). This suggests that *ZLL* alone is not sufficient to activate expression of the stem cell marker *CLV3*.

Next we investigated whether *ZLL* function affected the expression of *gWUS-GFP*$_3$. Initially, *gWUS-GFP*$_3$ expression was indistinguishable in wild-type (Fig. 4A,B) and *zll-1* (Fig. 4D,E) embryos until the late heart stage (see Table S3 in the supplementary material). From torpedo stage on, however, the *gWUS-GFP*$_3$ expression domain increased in size in *zll-1* as compared with wild type, and by late torpedo/bent cotyledon stage was strikingly broader (Fig. 4F; see Table S3 in the supplementary material). Similar results were obtained by in situ hybridisation and in some instances, *WUS* expression appeared to be displaced to the flanks of the meristem (Fig. 4F; see Table S4 in the supplementary material). The expansion of the *WUS* expression domain and the concomitant decrease of *CLV3* expression suggest that in *zll* embryos, WUS function is impaired and thus unable to maintain *CLV3* expression. To verify this, we analysed the effects of the *zll-1* mutation on ectopic *WUS* expression from the *Arabidopsis RIBOSOMAL PROTEIN SUBUNIT 5A* (*ATRPS5A*) promoter (Weijers et al., 2001). *ATRPS5A* directs expression predominantly to basal parts of the young embryo, to the whole embryo until torpedo stage and thereafter mainly to the meristem and cotyledons, at levels indistinguishable between wild type and *zll* (see Fig. S3 in the supplementary material). *ATRPS5A>>WUS* expression in a wild-type background led to stunted growth, ectopic outgrowths on the hypocotyl (Fig. 4H) and seedling lethality in the majority of cases (see Table S5 in the supplementary material), consistent with disturbed organ differentiation as reported previously (Brand et al., 2002; Gallois et al., 2002; Lenhard et al., 2002). These effects were suppressed by the *zll-1* mutation (Fig. 4, compare H with J; see Table S5 in the supplementary material), despite similarly high levels of *WUS* mRNA accumulation compared with the wild-type background (see Fig. S3 in the supplementary material), indicating that *ZLL* is necessary also for ectopic *WUS* function in the embryo.

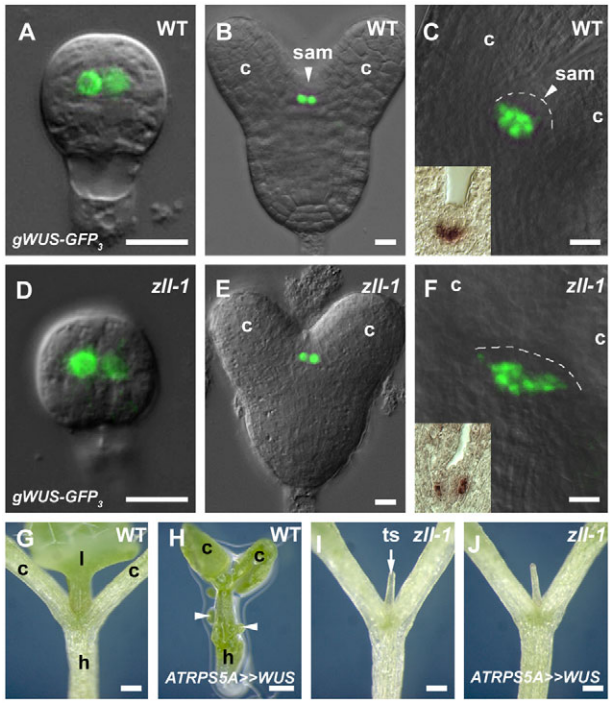

Fig. 4. *WUS* expression and function is altered in *zll-1* mutant embryos. (**A-C**) *gWUS-GFP*$_3$ expression in wild-type (WT) *Arabidopsis* embryo. (A) 16-cell stage showing WUS-GFP$_3$ (green). (B) Late heart stage embryo showing WUS-GFP$_3$ marking the organising centre. (C) Walking-stick stage embryo. The meristem dome is indicated with a dashed line. Inset shows endogenous *WUS* mRNA localisation in an embryo at the same stage. (**D-F**) *gWUS-GFP*$_3$ in *zll-1*. (D) 16-cell stage embryo. (E) Late heart stage embryo. (F) Walking-stick stage embryo showing a broad domain of *gWUS-GFP*$_3$ expression. The flat meristem dome is indicated with a dashed line. Inset shows endogenous *WUS* mRNA localisation at the same stage. (**G**) Wild-type seedling. (**H**) Wild-type *ATRPS5A>>WUS* seedling showing ectopic outgrowths on the hypocotyl (arrows) and stunted cotyledons. (**I**) *zll-1* seedling. (**J**) *zll-1 ATRPS5A>>WUS* seedling. All seedlings shown are at 11 days post-germination. c, cotyledon; sam, shoot apical meristem; h, hypocotyl; l, leaf; ts, terminal structure. Scale bars: 10 μm in A-F; 0.5 mm in G-J.

Taken together, these results suggest that *ZLL*-dependant signalling from the vascular primordium maintains stem cells during embryogenesis by potentiating *WUS* signalling from the OC to the stem cells. Since expansion of the *WUS* domain coincided closely with the stages when *pCLV3:GFP-ER* expression began to decrease, it is plausible that the failure to maintain *CLV3* function in *zll* embryos might in turn account for derepression of *WUS* transcription.

Because previous studies showed that the *ZLL* and *AGO1* genes share overlapping function during early embryogenesis (Lynn et al., 1999), we investigated whether AGO1 might also have a function in potentiating WUS signalling. Since double mutants between strong *zll* and *ago* alleles are embryo lethal, we generated *zll* mutants with partially altered levels of *AGO1* activity, similar to previous studies that considered flower and leaf development (Lynn et al., 1999). By reducing *AGO1* gene dosage to one functional copy, we found that stem cell defects in weak *zll-15* mutants were enhanced to the severity of the null allele *zll-1* without any other obvious effect on seedling development. In the complementary experiment, an increase of *AGO1* gene dosage to three copies reduced the severity of stem cell defects in *zll-1* (see Table S6 in the supplementary material).

Consistent with these data, *ago1-1* embryos showed defects in *gWUS-GFP*$_3$ expression similar to those observed in *zll-1*. The majority of homozygous *ago1-1* embryos showed an expanded and disorganised domain of *gWUS-GFP*$_3$ expression from as early as the transition to heart stage (see Fig. S4A and Table S7 in the supplementary material), and by maturity the domain was much broader than in segregating wild-type siblings (see Fig. S4B-D in the supplementary material). Unlike *zll-1*, however, most *ago1-1* embryos failed to initiate *pCLV3:GFP-ER* expression at the correct stage (see Fig. S4E and Table S8 in the supplementary material), despite strong expression of *gWUS-GFP*$_3$. Only at later stages of embryogenesis (see Fig. S4F and Table S8 in the supplementary material) did *ago1-1* embryos gradually recover *pCLV3:GFP-ER* expression to wild-type-like levels (see Fig. S4G,H and Table S8 in the supplementary material), coinciding with the approximate stage when ZLL becomes required for meristem maintenance. Therefore, whereas both ZLL and AGO1 appear to be required for normal expression of *WUS*, their affects on *CLV3* expression appear to be temporally separated. One plausible model is that AGO1 and ZLL act sequentially in stem cell development, with AGO1 being essential for initiation of the stem cell programme until heart/torpedo stage and ZLL for its maintenance during embryo maturation. In future studies it will be intriguing to determine whether AGO1, like ZLL, is a component of non-cell-autonomous signalling from provascular tissues during embryogenesis. Notably, *ago1* mutants display pleiotropic effects and are sometimes seedling lethal, indicating that AGO1 has additional functions that cannot be rescued by late ZLL expression.

Taken together, our results provide direct evidence that the vascular primordium, which is one of the first discernable tissues to form in the developing *Arabidopsis* embryo, plays an instructive role during embryonic stem cell development. This function is mediated by a *ZLL*-dependent signalling pathway, which, based on homology to AGO1 and recent studies of ZLL (Brodersen et al., 2008), is consistent with a role for small RNAs in this process. Since *pATHB8:YFP-ZLL* signal was not detectable outside of the provascular tissues during embryogenesis, it is likely that ZLL itself does not move and thus might be involved in the production or the transmission of a signal to the stem cell niche. The nature of this signal is currently elusive, but its central role in early vascular function makes the phytohormone auxin a plausible candidate to be tested in the future. As a consequence of this vascular-borne signal, *WUS* signalling from the OC is enabled to maintain the stem cells in an undifferentiated state. This could involve modulation of *WUS* function in the OC cells, or an effect on the competence of stem cells to respond to the *WUS*-dependent signal. Further studies aimed at elucidating the precise mechanism of ZLL function are necessary to distinguish between these models.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/17/2839/DC1

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