The ASH1-RELATED3 SET-Domain Protein Controls Cell Division Competence of the Meristem and the Quiescent Center of the Arabidopsis Primary Root

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The stem cell niche of the Arabidopsis (Arabidopsis thaliana) primary root apical meristem is composed of the quiescent (or organizing) center surrounded by stem (initial) cells for the different tissues. Initial cells generate a population of transit-amplifying cells that undergo a limited number of cell divisions before elongating and differentiating. It is unclear whether these divisions occur stochastically or in an orderly manner. Using the thymidine analog 5-ethyl-2'-deoxyuridine to monitor DNA replication of cells of Arabidopsis root meristems, we identified a pattern of two, four, and eight neighboring cells with synchronized replication along the cortical, epidermal, and endodermal cell files, suggested to be daughters, granddaughters, and great-granddaughters of the direct progeny of each stem cell. Markers of mitosis and cytokinesis were not present in the region closest to the transition zone where the cells start to elongate, suggesting that great-granddaughter cells switch synchronously from the mitotic cell cycle to endoreduplication. Mutations in the stem cell niche-expressed ASH1-RELATED3 (ASHR3) gene, encoding a SET-domain protein conferring histone H3 lysine-36 methylation, disrupted this pattern of coordinated DNA replication and cell division and increased the cell division rate in the quiescent center. E2Fa/E2Fb transcription factors controlling the G1-to-S-phase transition regulate ASHR3 expression and bind to the ASHR3 promoter, substantiating a role for ASHR3 in cell division control. The reduced length of the root apical meristem and primary root of the mutant ashr3-1 indicate that synchronization of replication and cell divisions is required for normal root growth and development.

Root growth depends on the continuous generation of new cells in the root tip. The root apical meristem (RAM) is composed of three main zones, determined by their cellular features, namely the stem cell niche (SCN);

the proximal meristematic zone (MZ), where cell division occurs; and the basal meristem, a transition zone (TZ) where cells start to elongate (Bennett and Scheres, 2010; Perilli and Sabatini, 2010; Perilli et al., 2012). The SCN comprises the quiescent center (QC; Fig. 1A), consisting of cells mitotically less active than the surrounding stem cells (initials). From the initials, daughter, granddaughter, and great-granddaughter cells are continuously produced in cell files of the MZ, ensuring a steady generation of new cells that will elongate and differentiate and thereby contribute to root growth and development (Jiang and Feldman, 2005; Moubayed et al., 2010; Perilli and Sabatini, 2010).

Exit from the MZ has been thought to coincide with a switch from the mitotic cycle to an endoreduplication cycle where the DNA is duplicated but no cytokinesis occurs (De Veylder et al., 2011). Recent evidence suggests that endoreduplication precedes rapid cell elongation (Hayashi et al., 2013). While proteins that control the switch to the endocycle have been described (De Veylder et al., 2011; Heyman and De Veylder, 2012), molecular components that control the maintenance of cell division in the MZ have not been identified to date. Like animals, plants control the entry into the S-phase of the cell cycle by the E2F-retinoblastoma pathway...
Retinoblastoma in animals and RETINOBLASTOMA-RELATED1 (RBR1) in Arabidopsis (*Arabidopsis thaliana*) are repressors of E2F transcription factors, and RBR1 overexpression results in the rapid loss of stem cell identity of root initials (Wildwater et al., 2005). Arabidopsis encodes three E2Fs (E2Fa, E2Fb, and E2Fc) that need to associate with one of two dimerization partners (DPa and DPb) to be active (Berckmans and De Veylder, 2009). E2Fa in association with DPa induces cell proliferation and increases ploidy levels (De Veylder et al., 2002). Likewise, E2Fb encodes an activator of cell proliferation, whereas E2Fc operates as a repressor (Magyar et al., 2005; del Pozo et al., 2006). E2F target genes have a cell cycle-modulated G1- or S-phase expression profile.

Deposition of appropriate epigenetic marks is necessary for the expression of cell cycle-related genes, for labeling of replication origins, and, during the S-phase, for maintenance of epigenetic signatures on new DNA double helices (e.g. signatures that will regulate gene expression and establish euchromatin and heterochromatin; Costas et al., 2011a; Dorn and Cook, 2011). SET-domain proteins represent important chromatin modifiers responsible for monomethylation, dimethylation, or trimethylation of various Lys residues on N-terminal histone tails (Kouzarides, 2007; Liu et al., 2010; Thorstensen et al., 2011). In a search for SET-domain proteins operating during the cell cycle, we identified the SCN-expressed Arabidopsis gene *ASH1-RELATED3* (*ASHR3*; Baumbusch et al., 2001). Here, we present genetic and molecular data indicating that *ASHR3* is a direct target for E2F transcription factors. *ASHR3* appears important for the maintenance of meristematic cell divisions based on a comparison of cell length and cell number in wild-type and *ashr3-1* MZ cell files: by monitoring DNA replication using the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU), a coordinated pattern of replication along the entire epidermal, cortical, and endodermal MZ cell files was revealed. Mitosis and cytokinesis...
markers displayed a similar pattern but were only present in two-thirds of the MZ, closest to the QC. The coordinated patterns of replication and cell divisions were distorted in the ashr3-1 mutant. Furthermore, in the ashr3-1 QC, we observed a higher number of cells and more cells undergoing DNA replication, suggesting that the quiescence of these cells is abrogated. Chromatin immunoprecipitation (ChIP) indicated that ASHR3 acts as a histone H3 Lys-36 (H3K36) monomethyltransferase to facilitate coordinated replication patterns in the root MZ.

RESULTS

The ashr3-1 Mutation Affects RAM Development and Organization

The Arabidopsis primary root displays a well-ordered symmetric pattern of cells in the RAM and the SCN (Fig. 1A). ASHR3 is one of the few genes encoding SET-domain proteins reported to be expressed in the QC and surrounding cells (Table I). The ASHR3 promoter-reporter construct pASHR3:GUS (Thorstensen et al., 2008) was expressed in the SCN region (Fig. 1A), thus confirming the microarray data (Nawy et al., 2005). The transfer DNA (T-DNA) insertion mutant ashr3-1, with more than 90% reduction in the ASHR3 transcript level (Supplemental Fig. S1, A and B), was inspected for aberrant root phenotypes. A growth assay showed that ashr3-1 roots were significantly shorter than wild-type roots 10 d after germination (DAG), when, under our growth conditions, the root meristem is fully developed (Fig. 1B). Introduction of a wild-type copy of the ASHR3 gene in the mutant restored the root length.

Anatomical measurements of confocal images of propidium iodide (PI)-stained roots disclosed significant differences in cell size, with more than 90% reduction in the cell size of mutant compared with the wild type. The frequency and distribution of neighboring replicating cells in the cortex (inspected in 16 cell divisions, Fig. 1C; Supplemental Fig. S1E). Introduction of the wild-type ASHR3 gene resulted in a cellular pattern and shortened MZs similar to those of the wild type (P = 0.064; Supplemental Fig. S1, E and F). The larger mutant cell size, which was also observed for differentiated mature cortex cells (Supplemental Fig. S1G), may explain the minor difference in MZ length and total length of young ashr3-1 roots.

Upon closer inspection of the SCN, we observed aberrant cellular organization and division planes in the ashr3-1 columella root cap (CRC) and the QC (Fig. 1E), a phenotype that also was rescued by complementation with the wild-type ASHR3 gene (Supplemental Fig. S1H).

The Pattern of Coordinated Replication Is Perturbed in the ashr3-1 Mutant

To substantiate an aberrant cell division phenotype, DNA replication in 10-d-old roots was monitored using a 1-h pulse treatment with EdU, a nucleoside analog of thymidine that is incorporated in DNA undergoing replication. The numbers and positions of replicating nuclei along the cortical, epidermal, and endodermal cell files were registered. Near the QC, about 10% of the cortical cells were replicating in the wild type, and after cell 16, 35% to 40% (Fig. 2A). In the ashr3-1 mutant, the replication activity was similar in the apical part of the meristem (cells 1–16) but lower than the wild-type level in the proximal part (cells 17–40; Fig. 2A).

In the wild type, we observed a nonrandom distribution of neighboring replicating cells along the cortical, epidermal, and endodermal cell files (Fig. 2B; Supplemental Fig. S2). The frequency and distribution of neighboring coreplicating cells in the cortex (inspected in 16 cell files, delimited to 40 cells) clearly diverged from a stochastic pattern. There was (1) an overrepresentation of two, four, and eight neighboring replicating cells and an absence or strong underrepresentation of triplets, quintets, sexets, and septets (Fig. 2C; Table II); (2) a spatial distribution with a region exclusively of singlets in the first few cells nearest the QC, with a subsequent rapid decline of singlets; (3) an abundance of duplets in cell positions five to eight; (4) a concentration of quartets in cells 17 to 20;

### Table I. Expression levels in the QC relative to the surrounding tissues of ASHR3 and selected genes with known QC expression

Table data were compiled from Nawy et al. (2005).

| Gene                  | QC Ratio* | Lateral Root Cap | CRC | Ground Tissue | Atrichoblasts | Stele |
|-----------------------|-----------|------------------|-----|---------------|---------------|-------|
| AT5G62165, AGAMOUS-LIKE42| 1.0       | 0.6              | 0.4 | 0.7           | 0.6           | 0.9   |
| AT4G30860, ASHR3        | 1.2       | 0.9              | 0.9 | 0.9           | 1.0           | 0.9   |
| AT3G20840, PLETHORA     | 6.3       | 1.2              | 2.8 | 1.2           | 0.7           | 0.9   |
| AT5G67260, CYCLIN D3;2  | 4.9       | 1.8              | 1.2 | 2.6           | 2.3           | 2.6   |

*Average ratio of the QC level and the level in the other tissues.
Figure 2. DNA replication and cell division patterns are distorted in ashr3-1 root meristems. A, Distribution of replicating cells along wild-type (Wt) and ashr3-1 cortical meristematic cell files of 10-d-old roots as percentages of total number of cells, starting from the stem cell and delimited to 40 cells. For the wild type, there were two sets with \( n = 8 \) cell files, from nine roots; for ashr3-1, there were two sets with \( n = 9 \) files, from 10 roots; total numbers of replicating cells are \( n = 206 \) (wild type) and \( n = 161 \) (ashr3-1), so values are indicated. B, Representative confocal images of primary root meristems of wild-type and ashr3-1 seedlings as indicated, incubated for 1 h with EdU. Examples of neighboring replicating cells, duplets (2), quartets (4), and octets (8), are indicated. Cells without replicating neighbors (singlets) were dominating in the boxed areas. Metaphases and anaphases are circled. C, Frequencies of singlets and categories of coreplicating cortical cells in wild-type and ashr3-1 cell files. D and E, Distribution of singlets and neighboring, coreplicating cells (duplets, quartets, and octets) along wild-type and ashr3-1 cortical cell files, as indicated, as percentages of the total number of replicating cells. For simplicity, triplets and sextets have been omitted. Compiled data for 16 wild-type cortical cell files from nine roots and 18 ashr3-1 cortical cell files from 10 roots are shown. F, Region with CYCLIN (CYC)B1;1-GUS expression in wild-type and ashr3-1 root tips. G, MZ of wild-type and ashr3-1 roots expressing CYCB1;1-GUS. Arrows indicate the end of the MZ. H and I, In situ immunolocalization of KNOLLE (green) in 3-DAG MZ. Counterstaining was done using PI (red). Single focus plane (H) and maximum projection (I) images were generated using Leica confocal software. Bars = 20 \( \mu \)m.
and (5) a rise in octets from cell 24 (Fig. 2D). Similar coreplication frequencies and patterns of distribution were seen for the endodermal and epidermal cell files (Table II; Supplemental Fig. S2, A–E); however, in the epidermis, both quartets and octets were abundant from cell 28 (Supplemental Fig. S2E). Coordinated replication of adjacent cells belonging to different cell layers was not observed.

In the *ashr3-1* mutant, the relative abundance of the categories of coreplicating cells in the cortex, epidermis, and endodermis deviated significantly from the wild type (Fig. 2C; Table II; Supplemental Fig. S2, B and C), as duplets in particular were overrepresented whereas octets were not found. Additionally, the frequency of quartets was reduced and/or the quartets appeared more distantly from the QC in *ashr3-1* than in the wild type (Fig. 2, D and E; Supplemental Fig. S2, D–G).

Interestingly, very few replicating cells were found present in the stle of the *ashr3-1* mutant line (Fig. 2B; Supplemental Fig. S2A), while at the same time, the number of cells in metaphase and anaphase were significantly higher than in the wild type (Fig. 2B; Table III; Supplemental Fig. S2A). Together, this suggested that loss of *ASHR3* interferes with cell cycle progression.

### Table II. Sister cell coreplication is dependent on *ASHR3*

The percentage of replicating cells that are single or coreplicating with neighboring cells is shown. The number of cells for each genotype was *n* = 348 (wild type) and *n* = 129 (*ashr3-1*) for the cortex, *n* = 348 (wild type) and *n* = 129 (*ashr3-1*) for the epidermis, and *n* = 265 (wild type) and *n* = 232 (*ashr3-1*) for the endodermal cell files.

| Category          | Cortex | Epidermis | Endodermis |
|-------------------|--------|-----------|------------|
|                   | Wild Type | *ashr3-1* | Wild Type | *ashr3-1* | Wild Type | *ashr3-1* |
| Singlets          | 10.7    | 10.9      | 19.3       | 24.1      | 13.2      | 14.6      |
| Duplets           | 20.4    | 36.3      | 28.7       | 46.6      | 24.4      | 37.8      |
| Triples           | 12.1    | 10.2      | 7.5        | 7.3       | 7.2       | 11.0      |
| Quartets          | 23.8    | 23.5      | 26.8       | 15.5      | 28.7      | 23.2      |
| Quintets and sextets | 11.2   | 19.1      | 7.1        | 2.6       | 7.2       | 13.4      |
| Octets            | 21.8    | 0.0       | 10.6       | 0.0       | 19.3      | 0.0       |
| Total             | 100     | 100       | 100        | 100       | 100       | 100       |

*χ*² test, *ashr3-1* versus the wild type: 0.001 1.4E-07 – 1.2E-05 – 1.4E-06

*χ*² test, versus wild-type cortex: 0.001 1.4E-16 0.29 2.7E-07

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### Cell Divisions Are Less Coordinated in *ashr3-1* Roots

To detect putative differences in mitotic activity, we monitored cell cycle progression by using a CYCB1;1-GUS reporter (Colón-Carmona et al., 1999) and cytokinesis by immune localization of the KNOLLE protein, a syntaxin involved in formation of the cell plate between separating sister cells (Lauber et al., 1997). Both approaches substantiated the role of *ASHR3* in coordination of the cell cycle in neighboring cells: in 6-DAG roots, the wild-type CYCB1;1-GUS was predominantly expressed in files of neighboring cells, while in *ashr3-1*, expression was characteristic to several single cells (Fig. 2, F and G; Supplemental Fig. S3A). KNOLLE was identified in neighboring cells undergoing cytokinesis in the wild type, while this was observed infrequently in images of in situ immune localization in *ashr3-1* 3-DAG root meristems (Fig. 2H; Supplemental Fig. S3B). Maximal projection of the confocal images suggested, in addition, that more cells underwent cytokinesis in the wild type versus *ashr3-1* (Fig. 2I).

In contrast to the DNA replication detected using EdU (Fig. 2A), neither CYCB1;1-GUS nor KNOLLE was localized in the entire MZ (Fig. 2, G–I). CYCB1;1-GUS protein was found in a region stretching from the QC to encompass 24.6 ± 3.3 wild-type and 26.3 ± 3.6 *ashr3-1* cortical cells (a nonsignificant difference; Student’s *t* test *P* = 0.22; *n* = 15). Similarly, in 3-DAG roots, KNOLLE localization was rarely found in the proximal third part of the MZ (Fig. 2, H and I; Supplemental Fig. S3B).

### A Functional *ASHR3* Gene Is Needed to Maintain QC Quiescence

Due to aberrant cellular patterning in the *ashr3-1* SCN (Fig. 1A), DNA replication in this region was studied in more detail. In the wild type, two to three QC cells can

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### Table III. *ASHR3* may be needed for efficient cell cycle progression

Number of metaphases (Meta) and anaphases (Ana) per root (*n* = 16 [wild type] and *n* = 10 [*ashr3-1*]) is shown in different tissues of the meristem. Two to three images were inspected per root.

| Plant | Stile Meta | Pericycle Meta | Endodermis Meta | Cortex Meta | Epidermis Meta | Cortex + Epidermis Meta | Total Meta |
|-------|------------|----------------|-----------------|-------------|----------------|------------------------|------------|
| Wild type | 0.3 0.0 0.1 0.0 | 0.2 0.0 0.6 0.0 | 0.2 0.0 0.6 0.0 | 0.2 0.0 0.6 0.0 | 0.2 0.0 0.6 0.0 | 0.2 0.0 0.6 0.0 | 1.0 |
| *ashr3-1* | 1.1 0.8 0.7 0.2 | 0.6 0.0 2.4 1.0 | 0.1 0.0 0.1 0.0 | 0.1 0.0 0.1 0.0 | 0.2 0.0 0.6 0.0 | 0.2 0.0 0.6 0.0 | 3.6 |
be observed in a single focus plane using confocal laser scanning microscopy and Nomarski optics with differential interference contrast (Fig. 3A). In contrast, in nine out of 10 cases, the ashr3-1 QC encompassed more cells, even up to 12 cells expressing QC-specific markers like GUS driven by the promoter of WUSCHEL-RELATED HOMEBOX5 (pWOX5:GUS) and QC46:GUS (Fig. 3, B and C). These cells were found near the stem cells of the endodermis/cortex and epidermis/lateral root cap (LRC; Fig. 3B). Consistent with this observation, a 1-h EdU pulse failed to capture the replication of wild-type QC cells but revealed replication in QC cells close to the endodermis/cortex and epidermis/lateral root cap initials in eight out of 10 ashr3-1 roots (Fig. 3, D and E).

ASHR3 Is a Target of E2Fa/E2Fb and Expressed during S-Phase

To substantiate a link to the cell cycle, we investigated whether the expression of ASHR3 was cell cycle phase dependent using synchronized Arabidopsis suspension culture cells. The majority of cells duplicate their DNA content from 2C to 4C within the first 4 h of the experiment. During this period, the ASHR3 transcript level peaked (Fig. 4A), showing that ASHR3 is an S-phase-regulated gene, confirming previous microarray data (Menges et al., 2003). The G1-to-S cell cycle transition is controlled by the E2Fa/E2Fb-DPa transcription factor complex. Consistent with the detection of E2Fa mRNA in the root meristem including the SCN (De Veylder et al., 2002), an E2Fa-GFP fusion protein, generated from a construct controlled by E2Fa’s own promoter, was found present in QC and initial cells as well as the MZ cells (Supplemental Fig. S4; Magyar et al., 2012). Therefore, we investigated whether ASHR3 was regulated by these transcription factors. In agreement with microarray data (Genevestigator [https://www.genevestigator.com/gv/plant.jsp]; Vandepoele et al., 2005), ASHR3 was strongly induced in transgenic lines overexpressing E2Fa-DPa (E2Fa-DPaOE), being about 30-fold more abundant than in wild-type seedlings (Fig. 4B). The ASHR3 expression level was reduced in an e2fa-2 but not an e2fb-1 mutant background; however, a strong reduction in transcript levels was observed in an e2fa-2 e2fb-1 double mutant, suggesting that E2Fa and E2Fb redundantly control ASHR3 expression similarly to their control of ULTRAVIOLET-B-INSSENSITIVE4 (UVI4; Fig. 4B; Heyman et al., 2011).

The ASHR3 promoter holds two potential consensus E2F-DP binding sites (−186 ATTGCCCC [reverse] and −123 TCTCCCGC; Vandepoele et al., 2005; Naouar et al., 2009). Binding of E2Fa-DPa to the latter sequence was demonstrated by electrophoretic mobility shift assay (EMSA) using a mutant version (TCTCC ATC) as a negative control (Fig. 4C). Furthermore, ChIP using anti-E2Fa antibodies resulted in an enrichment of an ASHR3 promoter fragment encompassing the putative E2F binding sites, at a level shown previously for the UVI4 promoter (Heyman et al., 2011), compared with the actin-negative control (Fig. 4D). These data show that E2F transcription factors can bind to the ASHR3 promoter and regulate its expression.

Figure 3. ASHR3 maintains QC quiescence. A and B, Representative wild-type (Wt) and ashr3-1 images, respectively, of SCN and surrounding tissue. Nuclei in the QC and stem cell layer of the CRC (layer D1) are indicated by black dots. C, Expression of the pWOX5:GUS and QC46:GUS QC marker lines in the wild type and ashr3-1 as indicated. Cor, Cortex; Epi, epidermis; Endo, endodermis; LRC, lateral root cap; Peri, pericycle. D and E, Representative images of wild-type and ashr3-1 SCN with 4′,6-diamino-phenylindole-stained nuclei (blue) after 1 h of treatment with EdU (green). Nuclei in the QC and CRC stem cell layers are indicated by red and white stars, respectively. F and G, Representative images of 7-d-old wild-type and ashr3-1 root tips, respectively, after Lugol staining of the starch granules in differentiated CRC cells. Nuclei in the QC and CRC stem cell layer (D1) are indicated by red and white stars, respectively.
ASHR3 Confers H3K36 Methylation

ASHR3 is a member of the ASH1/Set2 subgroup of histone Lys methyltransferases (HKMTases), which are associated with the transfer of methyl groups to H3K36, and in Arabidopsis, this subgroup encompasses four ASH1 HOMOLOG (ASHH1–ASHH4) genes and two additional ASH1 RELATED (ASHR1 and ASHR2) genes (Baumbusch et al., 2001; Thorstensen et al., 2011). To identify a link between mutant phenotypes and ASHR3’s expected function as an HKMTase, we performed ChIP experiments on 1-week-old seedlings using antibodies against H3K36 monomethyl (H3K36me1) and H3K36 dimethyl (H3K36me2). Irrespective of gene expression levels in the wild-type control and the ashr3-1 mutant background (Supplemental Fig. S5, A and B), all the genes tested, including housekeeping genes (ACTIN2 and TUBULIN8); genes encoding regulators of transcription (JASMONATE-ZIM-DOMAIN PROTEIN1 [JAZ1] and WRKY40); transcriptionally silent, late replicating transposable elements (Ta3, TSI, and CACTA); and genes reported to contain active replication origins in Arabidopsis cell cultures (ALLENE OXIDE SYNTHASE [AOS] and LIPOXYGENASE3 [LOX3]; Costas et al., 2011b), turned out to have reduced H3K36me1 levels (Fig. 5A). The H3K36me2 levels differed in general less between the wild type and the mutant, with the exception for WRKY40 and LOX3. Prominent ASHR3 expression has not been observed aboveground in seedlings (Thorstensen et al., 2008), and the lower H3K36 methylation level in ashr3-1 seedlings may predominantly be ascribed to a reduced expression level in the root cells where ASHR3 normally is expressed. This includes the SCN and, additionally, cells surrounding the base of emerged lateral roots (Thorstensen et al., 2008). ChIP on chromatin from inflorescences, where ASHR3 is strongly expressed in wild-type developing anthers (Thorstensen et al., 2008), also showed reduced H3K36me1 levels and, to some extent, H3K36me2 levels for the mutant (Supplemental Fig. S5), substantiating the link between ASHR3 and H3K36 HKMTase activity.

Figure 4. ASHR3 expression is cell cycle dependently regulated by E2Fa-DPa. A, qRT-PCR monitoring of ASHR3 transcript levels during the cell cycle in Arabidopsis cell suspensions synchronized using aphidicolin. Transcript levels were determined every second hour after aphidicolin removal. In parallel, the C value (haploid DNA content) of the cells was determined by flow cytometry. B, Relative expression level of ASHR3 determined by qRT-PCR in a line overexpressing E2Fa-DPa (De Veylder et al., 2002) and in 1-week-old e2fa-2 and e2fb-1 single and e2fa-2 e2fb-1 double mutant seedlings compared with the wild-type (Wt) level, which was set to 1. UVI4 was included as a positive control (Heyman et al., 2011). Data represent means ± se (n = 3). C, EMSA using in vitro-expressed E2Fa-DPa, a 32P-labeled oligonucleotide corresponding to an ASHR3 promoter fragment with a putative E2Fa-DPa binding site, and unlabeled competitor sequence with or without a two-nucleotide mutation. + indicates the presence and − the absence of a given ingredient in the reaction prior to gel electrophoresis. Stars indicate unspecific binding of the probe, and the arrow indicates the protein:DNA complex that can be competed with nonlabeled wild type but not with a mutant oligonucleotide. D, Enrichment of the promoter region encompassing the promoter fragment of ASHR3 used in C, relative to input after ChIP from 1-week-old seedlings with an E2Fa antibody and with a no-antibody control. The promoters of ACTIN2 and UVI4 were used as negative and positive controls, respectively.
Figure 5. ASHR3-dependent monomethylation and dimethylation of H3K36. ChIP analysis of wild-type (Wt) and ashr3-1 chromatin from seedlings was performed using antibodies against H3K36me1 (A) and H3K36me2 (B) as indicated. DNA levels from the experiment relative to the input reactions were quantified using qRT-PCR. Bars represent averages of two to three independent biological replicates, and so values are indicated.

DISCUSSION

ASHR3 Is a SET-Domain Protein Associated with H3K36 Monomethylation

The consistent reduction of H3K36me1 levels on genes and transposable elements in the ashr3-1 mutant advocates ASHR3 as the first SET-domain protein identified to date with histone H3K36 monomethyltransferase activity. The additional reduction in H3K36me2, an euchromatic mark associated genome-wide with transcribed genes (Oh et al., 2008), indicates that ASHR3 also may have dimethyltransferase activity and/or may provide H3K36me1 as a substrate for a dimethyltransferase. Hence, ASHR3 may mediate both transcription-independent H3K36me1 and transcription-associated H3K36me2. The residual H3K36me1 present at all loci tested suggests that there are more Arabidopsis HKMTases with H3K36me1 product specificity. Likely candidates are the ASHH and ASHR proteins, for which the specificity has not been determined yet (Baumbusch et al., 2001; Thorstensen et al., 2011). As the ashr3-1 single mutant displays a phenotype, these HKMTases are at most partially redundant with ASHR3 and may act in different cellular and epigenetic contexts.

Several lines of evidence argue that ASHR3 is a direct target of E2Fa/E2Fb transcription factors that control S-phase-dependent gene expression. Reduced ashr3-1 transcript levels in the ef2a-2 ef2b-1 double mutant indicate that ASHR3 might be redundantly regulated by E2Fa/E2Fb. In vitro EMSA confirmed binding of the promoter sequence postulated to have an E2F binding site. This was substantiated by ChIP and is also supported by a recent study where tandem chromatin affinity purification was used to identify targets of E2Fa (Verkest et al., 2014). This, together with cell cycle-regulated ASHR3 expression in cell culture, the ashr3-1 mutant root phenotype affecting replication and cell division, and, importantly, the overlapping expression patterns of E2Fa and ASHR3 in the SCN makes it conceivable that ASHR3 is part of the E2F-dependent regulation of RAM function.

Of the nearly 40 genes encoding SET-domain proteins in Arabidopsis (Baumbusch et al., 2001; Thorstensen et al., 2011), cell cycle regulation has been documented previously only for ARABIDOPSIS TRITHORAX RELATED5 (ATXR5) and ATXR6, encoding H3K27me1 HKMTases associated with the replication of heterochromatin (Raynaud et al., 2006; Jacob et al., 2009, 2010). As ASHR3 has predominantly eucharyotic localization and different HKMTase specificity, it is likely to have a function that differs from the ATXR genes. In fission yeast (Schizosaccharomyces pombe), H3K36 methylation has been implicated in replication fork stabilization and checkpoint activation (Kim et al., 2008). Arabidopsis origins identified in cell cultures have been reported preferably to reside in the 5' half of genes, to be enriched in histone H2A.Z, H3K4me2/H3K4me3, and H4K5 acetylation, and to be depleted in H3K4me1 and H3K9me2 (Costas et al., 2011b). We have identified transcription-independent ASHR3-dependent H3K36me1 both at such suggested origins of replication in the AOS and LOX3 genes and on other genes presumably farther away from origins (Costas et al., 2011b), but to date it is not known whether these origins are used in tissues of intact plants.

Daughter, Granddaughter, and Great-Granddaughter Cells in Meristematic Cell Files Exhibit ASHR3-Dependent Synchronized Replication

To monitor DNA replication in the MZ, we used a 1-h pulse with EdU, as a short EdU pulse facilitates the identification of cells replicating at the same time (Hayashi et al., 2013). Our EdU experiment revealed an ASHR3-dependent pattern where neighboring coreplicating cells preferentially come in duplets, quartets, and octets, with a nonrandom distribution along the MZ. To explain this pattern, we have developed a simple model suggesting that the progeny of each direct descendant (singlet) of a dividing epidermal, cortical, or endodermal stem cell will go through three to four consecutive synchronized rounds of cell division to give rise to duplets, quartets, and octets of coreplicating
neighboring daughter, granddaughter, and great-granddaughter cells along the MZ (Fig. 6A). Besides the pattern of distribution, the most compelling support for this model is the lack or underrepresentation of coreplicating triplets, quintets, sextets, and septets. The overrepresentation of quartets in the proximal part of the epidermal cell files, where the model predicts octets, can most likely be ascribed to differences between root hair cells (trichoblasts), reported to have the same length and cell division characteristics as cortical cells, and the longer nonhair cells (atrichoblasts; Beemster and Baskin, 1998). With a given length of the MZ, the fewer but longer atrioblasts may reach the TZ and exit from the MZ with one round of replication less than the trichoblasts and cortical and endodermal cells.

EdU incorporation showed that a normal frequency of DNA replication was dependent on ASHR3 both in the quartet and octet regions and confirmed that DNA replication takes place from the QC to the TZ, where cells start to elongate (i.e. in the entire region defined as MZ; Ubeda-Tomás et al., 2009). Synchronized mitosis and cytokinesis in neighboring cells also seem to be ASHR3 dependent, but in contrast to DNA replication, both the mitosis CYCB1;1-GUS and the cytokinesis KNOLLE markers (Ubeda-Tomás et al., 2009) demonstrated that cell divisions basically are confined to the first two-thirds of the defined MZ cells. The region with cell divisions (stopping at 24.6 ± 3.3 wild-type cortical cells) corresponds well to our simple working model, with on average four initials dividing into eight doublet daughter cells, giving rise to 16 quartet granddaughter cells that will replicate and divide: that is, 28 cells where cell division will occur (Fig. 6, A–C). Therefore, the last round of replication, seen in coreplicating octets of presumed great-granddaughters of dividing initials, is likely to be replication without cytokinesis, the process known as endoreduplication. This is also suggested from the large cells with big EdU-labeled nuclei near the TZ seen in our images (Fig. 6D) and described recently as 4C cells (Hayashi et al., 2013).

A key feature of our working model is the need for a mechanism that identifies cells of the same generation

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**Figure 6.** Working model for synchronized DNA replication and division of progeny cells along the cell files of the outer layers of the root MZ. A, DNA replication takes place in the whole MZ in an organized pattern. The first few cells that are direct progeny of the stem cell replicate uncoordinately as singlets and give rise to four pairs of daughter cells (duplets), for which sister cells will replicate coordinately. They, in turn, will give rise to four sets of granddaughter cells (quartets), each with coordinated sister cell replication, which subsequently results in four sets of coreplicating great-granddaughters. At the borders of singlets and duplets, triplets can accidently occur, and likewise sextets will arise between duplets and quartets. The arrowhead indicates the end of the MZ. B, The expression pattern of CYCB1;1-GUS suggests that mitosis only occurs in singlet, duplet, and quartet cells. C, Cytokinesis is also confined to singlets, duplets, and quartets, here demonstrated by the localization of KNOLLE-GFP on novel cell plates during the generation of quartets from duplets. D, DNA replication without cell division results in endoreduplication in the octet region, as also suggested by Hayashi et al. (2013) from the intensity of EdU staining, cell size, and the size of replicating nuclei. E, ASHR3 is needed for the coordinated DNA replication and cell division, as indicated by the absent cell walls in ashr3-1 (asterisks) compared with the wild type (see A). Theoretically, methylation of H3K36 by ASHR3 at specific targets in nuclei in the SCN, without renewal in the MZ cells, could work as a counter of cell divisions and an identifier of sister cells, as the level of the mark will be halved during each round of replication, providing each cell with a generation-specific and ASHR3-dependent H3K36 methylation level. This hypothesis remains to be tested.
so that they can be induced to behave in a coordinated manner. An epigenetic mark would be ideal for such a purpose, since without renewal during replication the level of a histone posttranslational modification (PTM) will be halved for each cell division (Hake and Allis, 2006). Incorporation of a PTM in chromatin of the QC and stem cells, followed by cell divisions without replenishment of the PTM, would in fact provide daughter, granddaughter, and great-granddaughter cells with their distinct levels of the given PTM (Fig. 6E), which would indicate how many cell divisions they have gone through and might facilitate synchronous replication. After three to four rounds of replication and cell divisions, the PTM level would be depleted (Fig. 6E) and fall below a needed threshold for coordinated behavior. This would coincide with cells reaching the TZ. Such a molecular mechanism, where dilution of a chromatin mark functions as a counter to monitor cell divisions, was recently demonstrated to function over a few cell divisions in the precise timing of termination of the Arabidopsis floral meristems (Sun et al., 2014; Zhang, 2014).

CONCLUSION

It remains to be investigated whether ASHR3-dependent H3K36 methylation can function as a cell generation mark or cell cycle counter in the root meristem (Fig. 6E). It is evident, however, that ASHR3 contributes to higher order synchronization of replication and cell division in the MZ. Two neighboring cells that grow at the same rate are likely to reach a size threshold at the same time and, therefore, replicate at the same time. But in ashr3-1 RAM cell layers, coreplicating duplets were overrepresented, and the frequencies of categories and the distribution of coreplicating cells deviated very strongly from the wild-type pattern (Table II). Additionally, neighboring cells expressing CYCB1;1-GUS or undergoing cytokinesis were underrepresented in the mutant compared with the wild type. Together, this resulted in shorter mature MZs and a reduction in total root length, indicating that such coordination contributes to normal root growth.

The reduced meristematic activity found in certain mutants (e.g. wox5 or ccs52a2) or overexpressor lines (e.g. CDC27α and CLE40), as a result of exogenous treatment with jasmonic acid, or by down-regulation of RBR1, has been associated with cell divisions in the QC, loss of stem cell activity followed by differentiation of RBR1, has been associated with cell divisions in the treatment with jasmonic acid, or by down-regulation of replication frequency of QC cells in the SHCC (Cruz-Ramírez et al., 2013). Intriguingly, the increased cell divisions in the ashr3-1 mutant are found at the flanks of the QC near the initials of outer cell files of the proximal meristem. In fact, QC cells may be considered a stem cell reservoir that can be activated upon damage of initial cells and be induced to divide more frequently under stressful conditions (Heyman et al., 2013). It was recently demonstrated that delayed S-phase entry significantly increased the frequency of QC divisions (Cruz-Ramírez et al., 2013). Hence, this may indicate that in the ashr3-1 mutant, loss of synchronized replication and reduced cell division rate are perceived as stress, leading to a release of the mitotic QC block as a secondary effect of the mutation.

MATERIALS AND METHODS

Plant Material and Constructs

Wild-type (Columbia-0) and transgenic Arabidopsis (Arabidopsis thaliana) plants were cultivated as described (Kumpf et al., 2013). The pASHR3::GUS construct, containing 2,054 bp of promoter (Thorstensen et al., 2008), was used to generate more transgenic lines. Consistently, these lines showed tapetum and lateral root expression as shown previously (Thorstensen et al., 2008) and expression in the SCN as described here. ashr3-1 mutant plants from the SAIL_804_D06 line are homozygous for a T-DNA insertion in the ASHR3 gene (At1g30680; Supplemental Fig. S1A). Wild-type plants segregating from this line were used as controls. The mutant phenotypes were complemented with a genomic fragment encompassing the promoter (see above) and the transcribed region of ASHR3 cloned in the T-DNA vector pGSC1704 (Supplemental Fig. S1).

The CycB1;1-GUS reporter (line FAAC) has 1.2 kb of promoter and coding sequences of the first 116 amino acids (including the mitotic destruction box) of CycB1;1 (At4G37490) in translational fusion with the uid gene encoding GUS (Colón-Carmona et al., 1999). The c2fb-2 (GABI_348E09, At1G36010) and c2fb-1 (SALK_103138, At5G22220) mutants were described previously (Berckmans et al., 2011a, 2011b), and the c2fb-2 c2fb-1 double mutant was generated by crossing (Heyman et al., 2011). The pE2Fa::E2Fa-GFP construct was described previously (Magyar et al., 2012).

Quantitative reverse transcription (qRT)-PCR to determine real-time transcription levels was performed essentially as described (Grini et al., 2009). Total RNA was extracted from 1-week-old seedlings using the RNeasy Kit (Qiagen). After DNase treatment with RNase-Free DNase (Promega), cDNA was synthesized using SuperScript II (Life Technologies) or the cDNA Synthesis Kit (Bio-Rad). Relative expression levels were determined with the LightCycler 480 Real-Time SYBR Green PCR System (Roche). For normalization, the genes At1G04850 and At5G18800, or ACTIN2 (At3G18780), CAK2 (At5G50000), and PAC1 (At1G22210), were used. U74V (At1G42260) was used as a positive control (Heyman et al., 2011).

EMSA

The E2Fa and DPa coding sequences, cloned into the pDEST17 and pGBKT7 expression vectors, respectively, were used as templates for protein expression using the Tnt Quick Coupled Transcription/Translation System (Promega). A mock reaction with an empty pDEST17 vector was used as a control. A total of 250 ng of the wild-type oligonucleotide 5'-aggagaaaccttccgggctgtaaalctt-3' with the putative E2Fa binding site (shown in capital letters) was labeled at the 5' end using [32P]ATP and T4 polynucleotide kinase (New England Biolabs). For the assessment of binding 200 ng of DPa and 300 ng of E2Fa, 2.5 ng of labeled probe, with or without 250 ng of unlabeled wild-type or mutant (5'-aggagaaaccttccgggctgtaaalctt-3') competitor, was incubated in 20 μl Tris, pH 7.5, 12.5% glycerol, 50 μM KC1, 1 μM MgCl2, 1 μM EDTA, and 0.2 μM dithiothreitol for 5 min at room temperature and for 30 min at 4°C. The reactions were run on a 5% polyacrylamide gel.
Flow Cytometric Analysis

Flow cytometric analysis was performed with Arabidopsis suspension cells synchronized with aphidicolin according to Porceddu et al. (2001).

Cell File Analyses

Roots were stained with 10 μm PI and images were taken with a confocal microscope (Zeiss LSM 710 and 780) and analyzed with the software Zen 2009 and ImageJ. A new plugin for ImageJ facilitating cell file analysis was provided by the Centre for Plant Integrative Biology.

GUS, EdU, and Lugol Staining

GUS assays were performed as described previously (Beeckman and Engler, 1994; Malamy and Benzly, 1997). GUS-stained seedlings were viewed using differential interference contrast optics as described previously (Beeckman and Engler, 1994). EdU staining of replicating DNA was performed according to the manual of the Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Invitrogen catalog no. A10202). Lugol solution was applied for 45 s to root tips, which were inspected immediately for staining of amyloplasts using Nomarski optics with differential interference contrast.

In Situ Immunolocalization

Three-day-old Arabidopsis seedlings were immunostained using anti-KNOLLE primary antibody (1:200 dilution) and Oregon Green-coupled anti-rabbit secondary antibody (Invitrogen catalog no. O6381; 1:200 dilution) as described previously (Péret et al., 2012). Counterstaining was done using PI (red). The images were taken using a Leica SP2 confocal microscope.

ChIP

ChIP to investigate the binding of E2Fa transcription factors to the ASHR3 promoter was performed with an anti-E2Fa antibody using 8- to 10-d-old plants according to the protocol of Berckmans et al. (2011) with minor adaptations. Pre-clearing of the extracted nucleosomes was performed using 40 μL of Dynabeads Protein G (Novex). After de-cross-linking, DNA was extracted using phenol-chloroform-isoamyl alcohol and purified using the MinElute PCR Purification Kit (Qiagen). As a negative control, immunoprecipitation was performed without any antibody. Quantitative PCR experiments were performed on equal concentrations of total input, no-antibody ChIP and E2Fa ChIP DNA, as measured with the Quant-iT dsDNA High Sensitivity Kit (Invitrogen). Primers used for PCR amplification were designed to amplify the ASHR3 upstream promoter region containing the conserved E2F cis-regulatory element using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/prime-blast/). UV14 and ACTIN were used as positive and negative controls, respectively (Heyman et al., 2011). Normalization of enrichment relative to total input DNA was calculated as $100 \times \frac{Ct(\text{Input})}{Ct(\text{ChIP})}$ (ChIP$\text{-}\text{input}$), where Ct is the threshold cycle.

ChIP for the detection of chromatin marks was performed as described (Veiseth et al., 2011) using 1-week-old seedlings or infflorescences and the antibodies anti-H3K36me2 (07-369; Upstate) and anti-H3K36me1 (ab9048; Abcam). A number of the genes tested, ALLENE OXIDE SYNTHASE (At5g26900), ALLENE OXIDE CYCLASE2 (At1g25780), JASMONATE-ZIM-DOMAIN PROTEIN1 (At1g19180), and WRKY40 (At1g80840), have been shown previously to be involved in auxin-dependent cell cycle reactivation through transcriptional regulation of Arabidopsis E2Fa by the ASHR3 locus.

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Supplemental Figure S3. E2Fa is expressed in the SCN.

Supplemental Figure S4. Expression levels of genes investigated by ChIP in roots.

Supplemental Figure S5. Genes investigated by ChIP in roots and infflorescences.

Supplemental Table S1. Primers.

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