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REVIEW

Lineage-specific stem cells, signals and asymmetries during stomatal development

Soon-Ki Han[1,2] and Keiko U. Torii[1,2,*]

ABSTRACT

Stomata are dispersed pores found in the epidermis of land plants that facilitate gas exchange for photosynthesis while minimizing water loss. Stomata are formed from progenitor cells, which execute a series of differentiation events and stereotypical cell divisions. The sequential activation of master regulatory basic-helix-loop-helix (bHLH) transcription factors controls the initiation, proliferation and differentiation of stomatal cells. Cell-cell communication mediated by secreted peptides, receptor kinases, and downstream mitogen-activated kinase cascades enforces proper stomatal patterning, and an intrinsic polarity mechanism ensures asymmetric cell divisions. As we review here, recent studies have provided insights into the intrinsic and extrinsic factors that control stomatal development. These findings have also highlighted striking similarities between plants and animals with regards to their mechanisms of specialized cell differentiation.

KEY WORDS: Asymmetric division, bHLH proteins, Epigenetic regulation of differentiation, Lineage-specific stem cells, Peptide signaling, Stomata

Introduction

Stomata are small cellular valves found on the epidermis of land plants that facilitate gas exchange while minimizing water loss. The correct density, distribution and differentiation of stomata are crucial for stomatal function and hence optimal plant growth and survival. A number of environmental and internal signals are known to influence stomatal patterning. Plant stomata have thus emerged as an excellent system for understanding how *de novo* lineage-specific stem cells initiate, proliferate and differentiate into specialized cell types, and how intrinsic polarity components and extrinsic signals enforce proper tissue patterning.

In the typical dicot plant, *Arabidopsis thaliana*, stomata are formed via a series of stereotypical cell divisions and cell state transitions (Fig. 1A). Stomatal progenitor cells emerge from a subset of protodermal cells as meristemoid mother cells (MMCs). An MMC undergoes an asymmetric entry division, giving rise to a meristemoid and its sister cell named a stomatal-lineage ground cell (SLGC). Meristemoids possess a stem cell-like character and reiterate asymmetric amplifying divisions, thereby maintaining the meristemoid while producing additional surrounding SLGCs. After a few rounds of asymmetric cell divisions (ACDs), meristemoids lose their stem cell-like potential and differentiate into round guard mother cells (GMCs), which divide symmetrically and terminally differentiate into paired guard cells (GCs) that surround a pore (Bergmann and Sack, 2007; Nadeau and Sack, 2002a; Pillitteri and Torii, 2012). As plant leaves expand, young SLGCs may

re-establish MMC identity and undergo asymmetric spacing division to form satellite (secondary) stomata. This occurs away from the existing stoma so that the two stomata are spaced by at least one cell (Fig. 1), a phenomenon known as the 'one-cell spacing rule' (Nadeau and Sack, 2002a). The rest of the cells in the epidermis differentiate into pavement cells, which protect plants from desiccation, pathogen invasion and other environmental insults.

Over the past decade, several key regulators of *Arabidopsis* stomatal development have been identified (Fig. 1A). Together, these findings have revealed that the cell fate transitions within the stomatal lineage are directed by the sequential actions of three master regulatory basic-helix-loop-helix (bHLH) transcription factors (TFs) – SPEECHLESS (SPCH), MUTE and FAMA – that drive the initiation, proliferation and differentiation of stomatal precursor cells (MacAlister et al., 2007; Ohashi-Ito and Bergmann, 2006; Pillitteri et al., 2007). These three bHLH proteins heterodimerize with two redundant partners, the SCREAM (SCRM; also known as ICE1) and SCRM2 bHLH TFs, that are expressed throughout the stomatal lineage and integrate the three steps of stomatal differentiation (Kanaoka et al., 2008).

The proper distribution and density of stomata are enforced by cell-cell signaling (Fig. 1). Notably, members of the EPIDERMAL PATTERNING FACTOR (EPF)/EPF-LIKE (EPFL) family of secreted peptides, which are perceived by three ERECTA (ER)-family leucine-rich repeat receptor kinases (LRR-RKs), ER, ER-LIKE1 (ERL1) and ERL2, and an LRR receptor-like protein, TOO MANY MOUTHS (TMM), restrict stomatal development (Hara et al., 2007, 2009; Hunt and Gray, 2009; Nadeau and Sack, 2002b; Shpak et al., 2005). This involves signaling via an intracellular mitogen-activated protein kinase (MAPK) cascade that includes YODA (YDA), MKK4/5 and MPK3/6 (Bergmann et al., 2004; Wang et al., 2007).

The identification of the above key regulators of stomatal development in recent years has moved research into new directions. The study of stomatal development has also revealed intriguing similarities between plants and animals in terms of how they generate specialized cell types, despite these two kingdoms having evolved multicellularity independently. In this Review, we aim to summarize the latest breakthroughs in stomatal development research and provide our perspectives. When possible, specific examples of cell differentiation pathways in plants and animals will be compared, in an attempt to deduce a conserved logic of developmental patterning.

Lineage specification and progression during stomatal development

Cell differentiation in many contexts is maintained by global transcriptional alterations mediated by a core network of transcription factors and epigenetic regulators. The bHLH family of TFs, in particular, appears to regulate lineage differentiation programs in various animal cell types. This includes the MyoD

[1]Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195, USA. [2]Department of Biology, University of Washington, Seattle, WA 98195, USA.

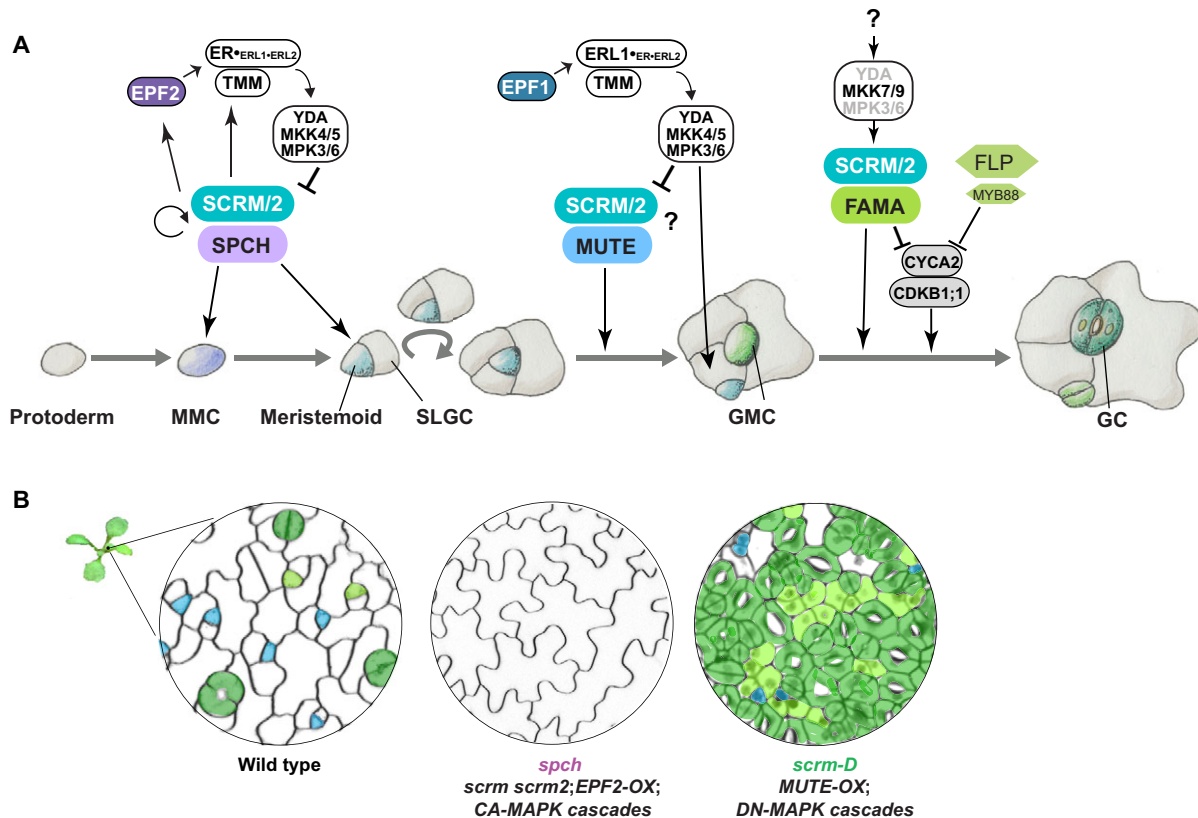*Author for correspondence (ktorii@u.washington.edu)

DEVELOPMENT

Fig. 1. Stomatal development in *Arabidopsis*. (A) Cell transitions and key regulatory pathways within the stomatal lineage. In the developing epidermis of photosynthetic tissues, an undifferentiated protodermal cell adopts a meristemoid mother cell (MMC, purple) identity and undergoes an asymmetric cell division (ACD), giving rise to a meristemoid (cyan). This initial step is directed by SPCH-SCRM protein heterodimers, which amplify their own expression while inducing the inhibitory secreted signals EPF2 and TMM. Signal transduction via ER family proteins and MAPKs, in turn, inhibits SPCH-SCRM, preventing neighboring cells from adopting a stomatal identity. The meristemoid reiterates ACDs, renewing itself and amplifying the surrounding stomatal-lineage ground cells (SLGCs, gray). A MUTE-SCRM module drives a meristemoid-to-guard mother cell (GMC, light green) transition, which terminates the stem cell-like state. EPF1 peptides, which signal via ERL1, TMM and the stomatal MAPK cascade, inhibit differentiation. The same pathway enforces the orientation of a secondary ACD of an SLGC, known as asymmetric spacing division. The transition from GMC to guard cell (GC, green) is specified by the FAMA-SCRM module. The MAPK cascade also promotes this step, although the upstream signal is unknown. FAMA and two paralogous MYB proteins, FLP and MYB88, restrict the GMC symmetric division by repressing cell cycle regulators such as *CDKB1;1* and *CYCA2*. (B) The loss or gain of function of key stomatal regulators can alter epidermal cell patterning. Shown are false-colored confocal microscope images of developing *Arabidopsis* epidermis from wild-type plants (left), *spch* mutants (middle) and *scrm-D* mutants (right). In *spch* mutants [as well as in *scrm scrm2* mutants, and in the case of *EPF2*-overexpression (OX) or constitutively active (CA) MAPK signaling], the epidermis is composed solely of pavement cells. Conversely, in *scrm-D* mutants [and in the case of *MUTE* overexpression (OX) and dominant-negative (DN) MAPK signaling], the epidermis is entirely composed of stomata. Cyan, meristemoids; light green, GMCs; green, GCs. Images are modified from Horst et al. (2015).

family in skeletal muscle myogenesis (Buckingham and Rigby, 2014), neurogenin and NeuroD in neurogenesis (Imayoshi and Kageyama, 2014) and E-proteins in lymphocyte development (Belle and Zhuang, 2014). In plants, too, closely related bHLH TFs mediate the consecutive steps of stomatal development. Similarities between the transcriptional regulatory networks controlling stomatal development in plants and myogenesis in animals have thus been indicated (Matos and Bergmann, 2014; Pillitteri and Torii, 2007). In addition, these comparisons have revealed that each cell state/fate-specific transition factor forms obligate heterodimers with broadly expressed common bHLH partners and binds to core E-box sequences.

Transcription factors regulating stomatal lineage specification
During muscle development in animals, a hierarchy of transcription factors regulates differentiation within the myogenic lineage (Bentzinger et al., 2012; Buckingham and Rigby, 2014). The homeoproteins sine oculis-related homeobox1 (Six1) and Six4, and the paired-homeobox transcription factors Pax3 and Pax7, are

upstream regulators that direct cells towards myogenesis. Subsequently, bHLH TFs and myogenic regulatory factors (MRFs), MyoD (Myod1) and Myf5, commit cells to a myogenic fate in a redundant fashion, whereas the MRFs myogenin and MRF4 (Myf6) control the differentiation of myoblasts into myofibers and myotubes (Bentzinger et al., 2012; Buckingham and Rigby, 2014). Likewise in plants, pre-specification of the shoot protodermal (L1) identity, which is controlled by the homeobox transcription factors MERISTEM LAYER 1 (ATML1) and HOMEODOMAIN GLABROUS 2 (HDG2), is required for the initiation of stomatal cell lineages (Takada et al., 2013; Peterson et al., 2013). The ectopic expression of ATML1 or HDG2 is sufficient to induce *SPCH* expression and subsequent stomatal differentiation in non-epidermal cells (Takada et al., 2013; Peterson et al., 2013). Loss-of-function *spch* mutants do not express any stomatal lineage markers and produce a leaf epidermis solely composed of pavement cells, whereas ectopic *SPCH* overexpression confers excess ACDs and generates highly divided small cells (MacAlister et al., 2007; Pillitteri et al., 2007).

A recent study has delineated how transcriptional regulation by SPCH contributes to the specification and proliferation of stomatal lineage cells (Lau et al., 2014). In this study, genome-wide SPCH targets were profiled by chromatin immunoprecipitation sequencing (ChIP-seq) and inducible *SPCH* transcriptome analysis. A stable, MAPK-insensitive version of SPCH (SPCH$_{1-4A}$) directly binds to the promoters of stomatal regulatory genes, including *SCRM*, *SCRM2*, *TMM*, *EPF2*, *ERL2*, *BASL* and *POLAR* (discussed below). Surprisingly, however, SPCH associates with roughly one third of *Arabidopsis* genes, and the majority of the binding sites are not directly linked to target gene expression (Lau et al., 2014). This suggests that SPCH binds to cis-elements with some affinity but requires other determinants for active transcription. Such widespread DNA binding is also observed for MyoD in ChIP-seq studies (Cao et al., 2010). The majority of MyoD binding sites in the two different cell states, myotube and myoblast, are identical, and they are inactive for enhancer function (Cao et al., 2010). In addition, MyoD binding leads to increased histone acetylation in the regions where it binds, reflecting the role of MyoD in establishing a muscle-specific open chromatin state (Cao et al., 2010). Hence, it would be interesting to analyze whether SPCH can also alter chromatin state during stomatal lineage initiation and, if so, how it affects the downstream factors leading to stomatal differentiation.

Termination of the self-renewing state and GC fate commitment

How a self-renewing meristemoid decides when to stop ACDs and proceed towards differentiation has been a long-standing question in the field. In the absence of subsequent MUTE activity, the meristemoid executes a prolonged ACD cycle, presumably owing to extended SPCH activity (Pillitteri et al., 2007). These two bHLHs therefore act functionally in opposing manners during stomatal development; whereas SPCH initiates entry into the stomatal lineage and ACD, MUTE terminates the stem cell-like activity of a meristemoid. In line with this, SPCH and MUTE exhibit overlapping but distinct expression patterns; SPCH expression diminishes in late meristemoids as MUTE expression appears (Davies and Bergmann, 2014). However, how SPCH and MUTE affect each other's expression remains unknown except that SPCH binds to the *MUTE* promoter (Lau et al., 2014) and that the HD-ZIP IV protein HDG2 can transactivate *MUTE* expression (Peterson et al., 2013).

Cell cycle regulators have also been implicated in the control of meristemoids; transcriptome profiling of meristemoid-enriched cell populations revealed enrichment of a number of core cell cycle regulators (Pillitteri et al., 2011). Furthermore, transcriptomic analyses of fluorescence-activated cell sorting (FACS)-isolated cells expressing *MUTE* revealed that cell cycle genes and genes associated with DNA and histone modifications are highly enriched in *MUTE*-expressing cells. Unique subsets of cell cycle genes (e.g. Cyclin D and APC/C family) are associated with *SPCH*- and *MUTE*-expressing cells, respectively, suggesting that specific cell cycle regulators drive a meristemoid ACD versus a GMC symmetric cell division (Adrian et al., 2015). These findings parallel those seen in myogenesis: the molecular characterization of MyoD and myogenin suggests that both factors play distinct roles in cell cycle modulation but act synergistically to drive muscle differentiation. For example, MyoD directly activates genes involved in cell cycle progression during myoblast proliferation, whereas myogenin induces anti-proliferative genes leading to cell cycle exit and myoblast differentiation (Singh and Dilworth, 2013).

Intriguingly, unlike *SPCH* or *FAMA* overexpression, which only produces more ACDs and single GCs, respectively, *MUTE* overexpression directs differentiation to intact mature stoma, including in the flower petal epidermis, which does not normally produce stomata (Pillitteri et al., 2008, 2007). This phenomenon is analogous to the trans-differentiation observed in animal cells (Iwafuchi-Doi and Zaret, 2014). For instance, ectopic expression of MyoD in fibroblasts induces muscle cell differentiation (Davis et al., 1987). The results of genome-wide MyoD binding studies indicate that MyoD binding requires specific E-boxes and is dependent on both interacting factors (cooperative or inhibiting) and a permissive epigenetic landscape (Fong et al., 2012). This might explain why MyoD exhibits limited ability to induce differentiation in some proliferating cells but can potentiate differentiation in heterologous cells, presumably owing to the presence of interacting partners or more accessible chromatin. Precisely how MUTE regulates the transcription of its target genes remains to be elucidated, although it has been found that MUTE functions without its DNA binding motifs (Davies and Bergmann, 2014). A plausible hypothesis, therefore, is that MUTE may be targeted to the genome by its dedicated partners, such as SCRMs, to cis-elements of target genes, for which chromatin architectures are epigenetically pre-determined in a lineage-specific fashion. This might explain why the ability of MUTE to cause trans-differentiation is constrained to epidermal cell types only (Pillitteri et al., 2008).

Terminal differentiation and maintenance of GC identity

The final step in stomatal development is mediated by FAMA, which promotes GC fate and restricts GMC symmetric cell divisions (Fig. 1A) (Ohashi-Ito and Bergmann, 2006). Accordingly, loss-of-function *fama* mutants undergo extra symmetric cell divisions that give rise to caterpillar-like cells. Further evidence for a role for FAMA in controlling GC fate has been provided by transgene studies (Lee et al., 2014a; Matos et al., 2014; Torii, 2015). For example, the introduction of an extra copy of epitope-tagged *FAMA* (FAMA^{trans}, *FAMApro::FAMA-GFP*) into wild-type *Arabidopsis* conferred the re-initiation of stomatal differentiation inside a stoma, a phenomenon termed stoma-in-stoma (SIS; Fig. 2). This phenotype is also observed when the RETINOBLASTOMA-RELATED PROTEIN (RBR) binding motif of FAMA was mutated, thereby abrogating the interaction between FAMA and RBR (Matos et al., 2014). This revealed a novel function of FAMA in maintaining GC identity via epigenetic control (discussed in detail below). In addition, microarray data from studies using an estradiol-inducible *FAMA* (Hachez et al., 2011) suggest that FAMA might directly activate differentiation genes but repress cell cycle control genes to restrict GC division. Thus, FAMA probably acts as both a transcriptional activator and a repressor.

Two R2R3 MYB transcription factors – FOUR LIPS (FLP) and MYB88 – restrict GMC division redundantly (Yang and Sack, 1995; Lai et al., 2005). FAMA and FLP/MYB88 exhibit similar expression patterns and function in a parallel pathway to regulate GMC cell division. Moreover, a *FLP* transgene (*FLPpro::FLP-GFP*) completely restores the *fama* mutant phenotype, indicating functional redundancy (Lee et al., 2014b). However, no physical interaction of FLP and FAMA has thus far been observed, suggesting that they do not work together as a TF complex (Ohashi-Ito and Bergmann, 2006; Lee et al., 2014b). CYCLIN DEPENDENT KINASE B1;1 (CDKB1;1) has been identified as a common target of FAMA and FLP/MYB88 (Xie et al., 2010; Hachez et al., 2011). Both proteins directly bind to the *CDKB1;1* promoter and repress *CDKB1;1* expression. *cdkb1;1/2* double mutants, *cyca2;2/3/4* triple mutants or dominant-negative *CDKB1;1* mutants all display single GCs, and such phenotypes
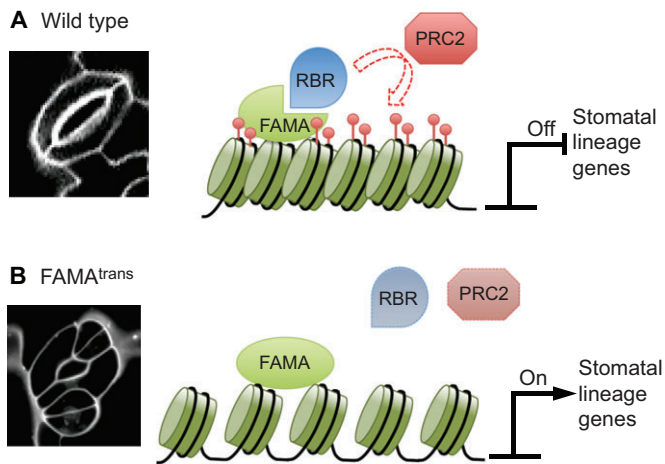
Fig. 2. A model for guard cell maintenance by epigenetic mechanisms.
(A) In the mature guard cells (GCs) of wild-type plants, stomatal lineage genes are terminally turned off. In this context, FAMA physically interacts with RBR via its LxCxE motif, which might recruit a PRC2 complex that deposits H3K27me3 marks (red circles) to give rise to a repressive chromatin state.
(B) When a GFP FAMA transgene or mutant FAMA lacking the LxCxE motif is introduced, stomatal lineage genes re-initiate their expression in mature GCs. In this case, RBR and the repressive complex are no longer recruited to target loci. As such, the chromatin state remains open, thereby resetting the stomatal differentiation program resulting in a stoma-in-stoma (SIS) phenotype. Confocal image in B was provided courtesy of Dr Eunkyoung Lee (University of British Columbia, Canada).

are epistatic to *flp myb88* (Xie et al., 2010; Vanneste et al., 2011). Furthermore, ChIP-on-chip experiments have identified additional cell cycle genes targeted by FLP/MYB88 (Xie et al., 2010).

Unlike *fama*, catapillar-like GMC tumors in *flp* or *flp myb88* double mutants eventually produce mature GCs, indicating a primary role of these two MYB genes in regulating GMC symmetric division (Lai et al., 2005). However, recent studies show that FLP is also required to maintain GC identity. Like FAMA[trans] plants, FLP[trans] (*FLPpro::FLP-GFP*) plants display a SIS phenotype (Lee et al., 2014a), and FLP physically interacts with RBR (Lee et al., 2014b). Thus, FAMA and FLP probably act redundantly to maintain GC identity in cooperation with RBR via an as yet unknown molecular mechanism.

The role of bHLH-interacting partners
In each stomatal lineage cell state, the actions of SPCH, MUTE and FAMA require obligate interactions with two paralogous bHLH transcription factors, SCRM and SCRM2, which are broadly expressed throughout the stomatal cell lineage (Kanaoka et al., 2008). Increasing loss of copy numbers of these two SCRM genes recapitulates the phenotype of *fama*, *mute* and *spch* mutants (Kanaoka et al., 2008). By contrast, the gain-of-function mutation of *SCRM* (*scrm-D*) produces a stomata-only epidermis, mimicking the *MUTE* overexpression phenotype (Fig. 1B), whereas loss of *MUTE* in *scrm-D* produces a meristemoid-enriched epidermis (Kanaoka et al., 2008; Pillitteri et al., 2011). The *scrm-D* phenotype is attributable to excessive stomatal lineage progression caused by stabilized SCRM activity (Horst et al., 2015; Kanaoka et al., 2008). A few SCRM targets, such as *SCRM* itself and *TMM*, are known to act in the initial step of stomatal cell lineages, during which both SPCH and SCRM directly bind to the promoter regions of their targets (Horst et al., 2015; Lau et al., 2014). How SCRMs associate with their target genes and their bHLH partners in different cell states remains to be determined. Nonetheless, this situation does

resemble that observed during myogenesis. During muscle development, the MRFs MyoD, myogenin and Myf5 sequentially associate with selected muscle-specific promoters. The binding of E-proteins, which cooperate with MRFs, often coincides with the MRFs, but does not entirely overlap with MRF binding during myoblast differentiation, indicating that they are independently recruited to the target genes (Londhe and Davie, 2011).

Epigenetic regulation of stomatal development
Emerging evidence has revealed that epigenetic, chromatin-based regulation, including DNA methylation, histone modification, nucleosome remodeling and regulation by non-coding RNAs, plays a central role in self-renewal, differentiation and reprogramming in both the animal and plant kingdoms (Fisher and Fisher, 2011; Han et al., 2015; Ikeuchi et al., 2015; Orkin and Hochedlinger, 2011). A number of epigenetic regulators have been implicated in the myogenic lineage program. For example, Pax transcription factors and MRFs are a frequent target of microRNAs (Gagan et al., 2012). In addition, MyoD directly binds to a chromatin remodeling complex component leading to chromatin remodeling and transcriptional activation (Forcales et al., 2012), and its binding to the E-box consensus motif at differentiation-specific genes is actively excluded in proliferating myoblasts by a chromatin repressive complex (Soleimani et al., 2012). Histone chaperone activity is also required for myogenic differentiation, recruiting histone demethylase to the repressive histone mark H3K27me3 deposited by PRC2 to activate transcription (Wang et al., 2013). Combined, these data suggest that the binding of transcription factors to their specific targets is dynamically regulated, allowing unique transcriptional programs to be activated in different cell types based on the presence of chromatin regulators or chromatin accessibility.

Key roles for DNA methylation and the transcriptional silencing of stomatal-lineage genes have been reported. Genome-wide DNA methylome data revealed that *YDA*, *EPF2* and *SCRM* are hypermethylated in their gene bodies and these modifications are lost in *met1* (DNA methyltransferase 1) mutants, whereas *SPCH*, *MUTE* and *FAMA* are found to be unmethylated (Cokus et al., 2008). In addition, studies of *Arabidopsis* plants grown under low relative humidity (LRH), which reduces the number of stomata, show that they exhibit *de novo* cytosine methylation and transcriptional repression at the *SPCH* and *FAMA* loci (Tricker et al., 2013). LRH-induced DNA methylation is abolished in DNA methylation enzyme mutants. Such mutants do not display an LRH-triggered reduction in stomatal numbers (Tricker et al., 2013). It has also been shown that REPRESSOR OF SILENCING1 (ROS1; also known as DEMETER-LIKE 1) mediates active DNA demethylation on the *EPF2* promoter and upstream transposable element (Yamamuro et al., 2014). In line with this, loss-of-function *ros1* and two other DNA demethylase mutants produce excess stomatal lineage cells. This phenotype is suppressed by mutations that affect RNA-directed DNA methylation (RdDM) and correlates with *EPF2* expression (Yamamuro et al., 2014). It remains unclear whether *EPF2* expression is conditionally regulated by DNA methylation in a wild-type background. However, overall the combined data suggest that active DNA methylation and demethylation impact the initiation, maintenance and differentiation of stomatal lineage cells by modulating the expression of key regulatory factors in the pathway.

Epigenetic regulators have also been implicated in maintaining cell fate within the stomatal lineage (Fig. 2). The FAMA-RBR module, for example, is required to maintain GC identity even

DEVELOPMENT

though FAMA and RBR are not expressed in mature stomata (Lee et al., 2014b; Matos et al., 2014; Ohashi-Ito and Bergmann, 2006). FAMA binds to RBR via an LxCxE motif, and both proteins bind directly to stomatal lineage genes and may recruit repressive complexes, such as Polycomb repressive complexes (PRCs), leading to the transcriptional repression of these genes (Matos et al., 2014). The ectopic expression of CLF, a PRC2 complex component that catalyzes H3K27me3, in FAMA[trans] plants suppresses the SIS phenotype. However, *swn clf* double mutants [in which CLF and another PRC2 component, SWINGER (SWN), are knocked out] develop intact stomata and do not display SIS phenotypes despite their pleiotropic developmental phenotypes (Lee et al., 2014a). Moreover, inconsistent expression patterns of *SPCH* and *MUTE* (i.e. a reduction in *SPCH* and an increase in *MUTE*) are observed in *swn clf* mutants (Lee et al., 2014a). Thus, it is not clear which PRC2 components regulate GC identity, or how they do so, together with the FAMA-RBR module. The PRC2 complex can also recruit the PRC1 complex via H3K27me3 marks (Merini and Calonje, 2015; Mozgova and Hennig, 2015), and a more recent model proposes that PRC1 activity is required for PRC2 recruitment (Merini and Calonje, 2015). Additional gene silencing mechanisms might, therefore, act together with the PRC2 complex to control the expression of stomatal lineage genes.

Cell-cell signaling enforces proper stomatal patterning

Cell-cell communication via ligand-receptor systems plays an essential role in development and tissue patterning in animals. Like animals, plants use a plethora of secreted peptide signals to coordinate growth and development (Fukuda and Higashiyama, 2011; Lee and Torii, 2012). In the case of stomatal development, secreted peptides belonging to the EPF/EPFL family of cysteine-rich peptides enforce proper stomatal patterning. Their receptors thus far identified include three ER-family receptor kinases (ER, ERL1, ERL2) and the receptor-like protein TMM.

The EPF/EPFL family protein EPF2 is expressed in early precursors, MMCs and early meristemoids, and *epf2* mutation results in excessive numbers of small stomatal-lineage cells (Hara et al., 2009; Hunt and Gray, 2009). EPF1, by contrast, is expressed in later precursors, late meristemoids and GMCs, and *epf1* mutation confers adjacent stomata (Hara et al., 2007). Using dominant-negative forms of ER-family receptor kinases, it was deciphered that the EPF2-ER ligand-receptor pair primarily restricts the initiation of stomatal cell lineages, whereas the EPF1-ERL1 pair primarily enforces stomatal spacing and represses GC differentiation *in vivo* (Lee et al., 2012). It should be noted, however, that the overexpression of these components *in planta* revealed that they do exhibit some promiscuity (Jewaria et al., 2013). Consistent with the genetic interactions, EPFs bind the ectodomain of ER-family receptors, and ER-family members and TMM form hetero-oligomers (Lee et al., 2012).

In contrast to EPF1 and EPF2, STOMAGEN (also known as EPFL9) promotes stomatal differentiation (Hunt et al., 2010; Kondo et al., 2010; Sugano et al., 2010). *STOMAGEN* overexpression results in increased numbers of stomata that are often clustered and, conversely, its antisense suppression reduces the number of stomata (Sugano et al., 2010). Interestingly, *STOMAGEN* is expressed in the internal layers of leaves, which will differentiate into a photosynthetic mesophyll tissue (Kondo et al., 2010; Sugano et al., 2010). The expression patterns and functions of *STOMAGEN* imply that a mobile signal from the developing photosynthetic tissue can induce the formation of stomata from where carbon dioxide for photosynthesis can be readily acquired. A recent study

showed that EPF2 and STOMAGEN bind to the same receptor, ER, in a competitive manner with a similar affinity (Lee et al., 2015). Whereas EPF2 activates downstream signaling, STOMAGEN does not. This competitive interaction may fine-tune proper stomatal density and patterning.

The pathways activated downstream of these ligand-receptor interactions have also been investigated, highlighting key roles for the MAPK cascade. EPF-ER-family/TMM signaling is mediated by YDA, MKK4/5 and MPK3/6 (Bergmann et al., 2004; Wang et al., 2007). Loss-of-function mutations in these components confer an epidermis that is solely composed of stomata, a phenotype that is identical to that seen following *scrm-D* or *MUTE* overexpression, and, conversely, the constitutive activation of these MAPK cascade components results in a pavement cell-only epidermis resembling the *spch* phenotype (Fig. 1). The signaling intermediates between the receptors and these MAPK components are unknown. In addition, a MAPK phosphatase, AP2C3 (also known as PP2C5), is expressed in stomatal lineage cells and when overexpressed confers an epidermis that is solely composed of stomata, suggesting that it might act as a negative regulator of stomatal signaling (Umbrasaite et al., 2010). However, *ap2c3* loss-of-function mutants do not exhibit any phenotype (Umbrasaite et al., 2010), implying possible redundancy or, alternatively, that endogenous AP2C3 does not play a direct role in stomatal development. In animals, MAPK cascades play pivotal roles in the regulation of cell proliferation, differentiation, and tissue patterning (Qi and Elion, 2005). Furthermore, studies of neurogenesis have established a direct connection between the MAPK cascade and bHLH proteins (Bain et al., 2001). Similarly, during stomatal development, SPCH is directly phosphorylated by the MPK3/6 (Lampard et al., 2008), further emphasizing the striking parallels between the two kingdoms.

Studies have also shown that EPFs signal differentially to modulate stomatal development. EPF2 application gives rise to a pavement-cell-only epidermis, resembling that seen in *spch* mutants, whereas EPF1 application results in arrested meristemoids, as observed in *mute* mutants (Hara et al., 2007, 2009; Lee et al., 2012). This suggests that EPF2-ER and EPF1-ERL1 signals primarily target the cell-transitional steps mediated by SPCH and MUTE, respectively. Consistently, the expression of constitutively active forms of MKK4 and MKK5 driven by the *SPCH* and *MUTE* promoters resulted in phenotypes that resembled the *spch* and *mute* phenotypes, respectively (Fig. 1) (Lampard et al., 2009). These observations point to a molecular framework in which the regulatory circuits between signaling components and bHLH TFs constitute a molecular switch that controls initiation and termination of the stomatal precursor stem-cell state.

Such a circuitry has been demonstrated for the initiation of stomatal cell lineages (Horst et al., 2015; Lau et al., 2014). First, SPCH and SCRM constitute a positive-feedback loop, whereby SPCH and SCRM upregulate *SCRM* expression in the protoderm. SPCH directly binds to the promoters and activates the expression of *EPF2* and *TMM*. These, together with ER-family receptors, transduce signals to inhibit SPCH-SCRM accumulation, thereby forming a negative-feedback loop. Although the promoter activity of *SPCH* is uniform throughout the protoderm, this feedback regulation limits the numbers of stomatal precursors and ensures their uniform distribution. Mathematical modeling predicts that an additional negative-feedback loop is necessary to explain all the available mutant and transgenic stomatal phenotypes (Horst et al., 2015). Components of the brassinosteroid (BR) signaling pathway (discussed below) may be candidates for this additional

loop, and some BR biosynthesis and signaling genes are direct targets of SPCH (Lau et al., 2014). Based on these studies, an MMC can be redefined as a cell that accumulates SPCH and SCRM above a certain threshold.

Signal integration during stomatal development

Stomatal density and distribution impact plant growth and productivity, so it is not surprising that numerous endogenous hormones as well as environmental inputs, including both abiotic and biotic stresses, influence stomatal development (Casson and Gray, 2008; Pillitteri and Torii, 2012). It is therefore of interest to the broader community of plant biologists to delineate how multiple signaling pathways are integrated into the core stomatal differentiation programs and signal transduction pathways (Fig. 3).

Integrating brassinosteroid and effector signaling into the core stomatal pathway

The molecular intersection between the stomatal and BR signaling pathways has been studied extensively. BRs are plant steroid hormones that regulate growth, development and fertility (Clouse and Sasse, 1998). In response to BR binding, the BR receptor, BRASSINOSTEROID INSENSITIVE 1 (BRI1), binds to its heterodimeric partner, BRI1-ASSOCIATED RECEPTOR KINASE (BAK1, also known as SERK3). This facilitates the interaction of a receptor-like cytoplasmic kinase, BR SIGNALING KINASE 1 (BSK1) and a phosphatase, BRI1 SUPPRESSOR 1 (BSU1). BSU1 dephosphorylates the key negative regulator of BR signaling, BRASSINOSTEROID INSENSITIVE 2 (BIN2), which is a

member of the GSK3/Shaggy-family of protein kinases. In the absence of BR input, BIN2 phosphorylates the transcription factors BES1 and BZR1 to prevent BR-mediated gene expression. In the presence of BR input, BSU1 inactivates BIN2, thereby enabling BES1 and BZR1 to promote target gene expression (Fig. 3) (Belkhadir and Chory, 2006; Kim and Wang, 2010).

The *bri1* mutant exhibits mild stomatal pairing, which implies a potential role for BR perception in proper stomatal patterning. In addition, the higher-order quadruple mutants of *BSU1* and its paralogs, *bsu-q*, exhibit severe stomatal clustering, nearly resembling the *scrm-D* phenotype (Kim et al., 2012). The downstream TFs of the BR signaling pathway, BZR1 and BES1, do not influence stomatal patterning. Instead, genetic and pharmacological dissections revealed that the GSK3-like kinase BIN2 acts downstream of ER-family receptors and TMM but upstream of YDA and SCRM to mediate the effects of BR (Fig. 3) (Kim et al., 2012). Further biochemical studies showed that BIN2 associates with and phosphorylates the N-terminal auto-inhibitory region of YDA and inhibits its activity. MKK4 and MKK5, two MAPK kinases (MAPKKs) that are immediately downstream of YDA, have also been shown to interact with and be phosphorylated by BIN2 *in vitro* (Khan et al., 2013). This phosphorylation appears to be specific to MKK4 and MKK5, as BIN2 fails to phosphorylate MKK7, a MAPKK that can promote stomatal differentiation if ectopically expressed in GMCs (Lampard et al., 2009). Overexpression of the mutant MKK4 harboring disrupted BIN2-target sites conferred constitutive stomatal differentiation, supporting the notion that BIN2 inhibits MKK4/5 (Khan et al.,
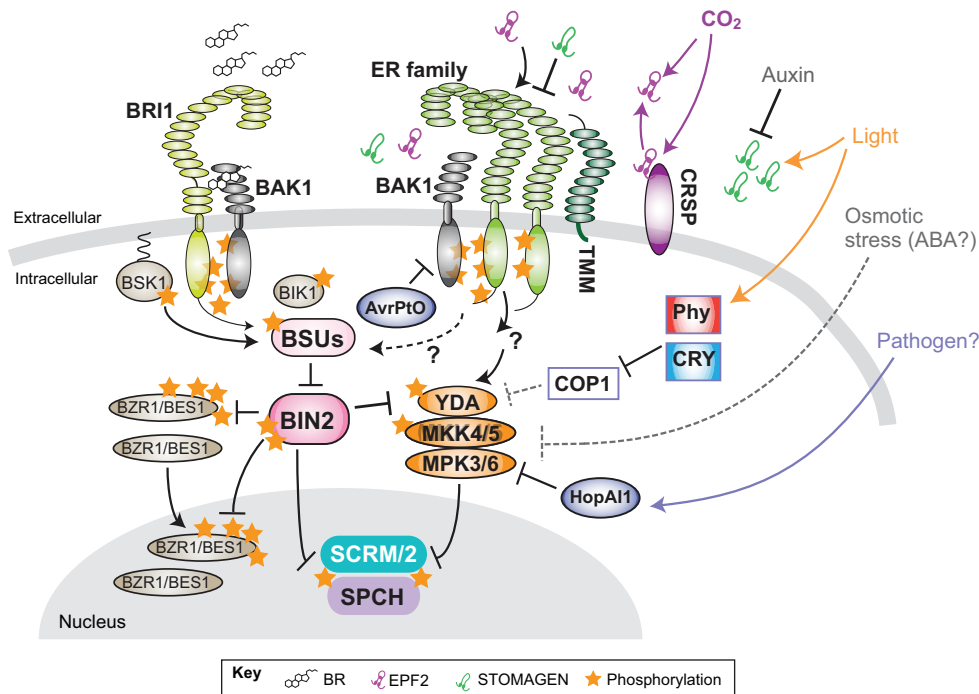

Fig. 3. The integration of environmental and hormonal signals during stomatal development. During stomatal development, EPF peptides are perceived by ER family (green) and TMM (dark green) receptor complexes, which also include a receptor mediator BAK1 (gray). The actual architecture of these receptor complexes is unknown. The signal is mediated via a MAPK cascade (orange), involving YDA, MKK4/5 and MPK3/6, that inhibits SPCH-SCRM via direct phosphorylation. Signaling via the plant steroid hormone brassinosteroid (BR) is mediated by the ligand-triggered heterodimerization of BRI1 (light green) and BAK1 (gray), which activates the BSU1 phosphatase that inhibits BIN2, a downstream negative regulator of BR signaling. BIN2 inhibits not only the BR downstream TFs BZR1 and BES1, but also YDA, MKK4/5 and SPCH to modulate stomatal development. Pathogen effector signaling, via AvrPtO and HopAI1 (marine blue), probably interferes with stomatal development by suppressing the activities of BAK1 and MPK3/6, respectively. Light, auxin and CO_2 induce or suppress STOMAGEN or EPF2, thereby altering the balance of positive and negative signals instructing stomatal differentiation. For example, elevated CO_2 induces the expression of CRSP, a protease that cleaves the EPF2 prepeptide. Light signals perceived by phytochrome (PHY) and cryptochrome (CRY) photoreceptors probably attenuate the stomatal MAPK cascade acting via COP1.

2013). These two studies suggest that BIN2 associates with and inhibits the activities of the YDA-MKK4/5-MPK3/6 complex as a whole, and this probably represents the molecular basis of BR signal integration during stomatal development (Fig. 3).

Although these reports suggest that BRs inhibit stomatal development, a separate study has suggested that BR signaling can positively regulate stomatal differentiation. In this study, a loss-of-function mutation in the gene encoding the key BR biosynthetic enzyme CPD was shown to confer reduced stomatal development in *Arabidopsis* hypocotyls (Gudesblat et al., 2012). Following on from this, it was demonstrated that BIN2 directly phosphorylates SPCH *in vivo*, leading to SPCH destabilization. Consistently, the removal of BIN2-target sites from SPCH conferred excessive asymmetric entry divisions (Gudesblat et al., 2012). These seemingly contradicting roles of BR in stomatal development might be due to differences in the organs studied. Alternatively, they might reflect complex inter-connected regulatory relationships between the BR and stomatal pathways, as also revealed by the genome-wide profiling of SPCH direct targets (Lau et al., 2014) and by a SPCH-SCRM regulatory circuit modeling study (Horst et al., 2015). In addition to BRs, dysfunction in sterol biosynthesis confers stomatal clustering (Qian et al., 2013), but the underlying molecular mechanism remains unknown.

The phenotypic discrepancy between the mild stomatal pairing in *bri1* and *cpd* and that of the severe stomatal clustering in *bsu-q*, however, imposes a question of whether these BSUs actually represent the downstream components of the BR pathway. Rather, it is more reasonable to hypothesize that some BSUs act downstream of ER-family/TMM signaling to directly influence stomatal patterning. Although experimental evidence is not available, a recent study has established a compelling role for BAK1 in enforcing stomatal patterning (Meng et al., 2015). This finding was made through the fascinating observation that the induced expression of the bacterial pathogen *Pseudomonas syringae* effectors *AvrPtO* and *AvrPtoB* in *Arabidopsis* seedlings confers severe ectopic stomatal differentiation (Meng et al., 2015). These effectors are known to target BAK1 and other SERK-family receptor kinases (Fig. 3) (Shan et al., 2008). Moreover, higher-order *bak1/serk* mutants recapitulated the severe stomatal clustering phenotype, resembling that seen in *er erl1 erl2* triple mutants. Importantly, the *bak1-5* allele, which has no effects on BR signaling (Schwessinger et al., 2011), still conferred severe stomatal clustering in the absence of SERK1/2 (Meng et al., 2015). Consistent with these phenotypic data, BAK1 and ER-family can trans-phosphorylate each other, and BAK1 physically associates with ER-family receptors in an EPF ligand-dependent manner. BAK1, in addition, physically associates with TMM, but in an EPF-independent manner, implying that BAK1 and TMM form a pre-activated complex (Fig. 3) (Meng et al., 2015). How BAK1 transduces EPF signals remains unknown, but this study unravels yet more complexity in stomatal signaling at the level of shared co-receptors (Chinchilla et al., 2009).

The effects of AvrPtO and AvrPtoB on stomatal clustering also highlight pathogen effector-mediated control of stomatal development. In addition to these effectors, the chemical induction of HopA1, a *P. syringae* effector specifically targeting MPK3/6 (Zhang et al., 2007), confers severe stomatal clustering phenotype (Fig. 3) (Kim et al., 2012). *P. syringae* invades hosts through stomatal pores (Melotto et al., 2006). Thus, an intriguing hypothesis is that the pathogen produces effectors that increase the number of stomata, thereby increasing entry sites for infection. Addressing such a hypothesis would require careful examination of stomatal lineage marker dynamics upon pathogen infection. Alternatively, because the targets of these effectors, BAK1/SERKs and MPK3/6, mediate the core immunity response (Chinchilla et al., 2009; Meng and Zhang, 2013; Tena et al., 2011), excessive stomatal differentiation might simply reflect a collateral consequence due to inactivation of danger signaling. Future studies may clarify the intersection of signaling pathways mediating stomatal patterning and immunity.

Osmotic stress and abscisic acid
Drought and osmotic stress reduce stomatal development. Although osmotic and abscisic acid (ABA) stress hormone signaling pathways have been studied extensively (Fujita et al., 2013; Lee and Luan, 2012), their integration points within stomatal pathways are only just beginning to be unraveled. Osmotic stress reduces the number of MMCs, as evidenced by the reduced expression of *EPF2pro::GFP* due to destabilization of SPCH-GFP protein (Kumari et al., 2014). This decrease in MMCs occurs independently of the EPF-ERECTA family/TMM ligand-receptor system, but via a MAPK cascade (Fig. 3) (Kumari et al., 2014). Consistently, the MAPK-insensitive mutant version of SPCH no longer responds to osmotic stress (Kumari et al., 2014). Other studies have further emphasized the role for MAPK cascades as a signaling hub (Meng and Zhang, 2013; Tena et al., 2011; Xu and Zhang, 2015).

Whether the repression of stomatal development by osmotic stress is mediated by ABA, a master stress hormone, is an interesting question. An ABA-deficient mutant, *aba2*, exhibits increased numbers of stomata, which is accompanied by prolonged duration of meristemoids; ABA application to these mutants reduces the number of stomata (Tanaka et al., 2013). *aba2 mute* double mutants dramatically increase their numbers of arrested meristemoids and surrounding SLGCs, suggesting that ABA loss promotes asymmetric entry and amplifying divisions. This is probably due to excessive activity (or stability) of SPCH, as *spch* mutation is epistatic to *aba2* (Tanaka et al., 2013).

Signal integration controlling upstream signaling ligands
As mentioned above, proper stomatal patterning is fine-tuned by the competitive actions of EPF2 and STOMAGEN (Kondo et al., 2010; Lee et al., 2015; Shimada et al., 2011; Sugano et al., 2010). Therefore, it is plausible that environmental and hormonal inputs influence stomatal patterning by directly changing the balance between these two antagonistic peptides. Indeed, *STOMAGEN* expression is upregulated by light, which probably contributes to light-regulated increases in stomatal numbers (Fig. 3) (Hronkova et al., 2015). Genetic studies suggest that light signals perceived by phytochromes and cryptochromes are mediated by CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), then merge into the YDA-canonical stomatal pathways (Fig. 3) (Casson et al., 2009; Kang et al., 2009). The phytochrome-interacting bHLH protein PIF4 also affects stomatal development (Casson et al., 2009), whereas the phytohormone auxin inhibits stomatal differentiation, possibly via direct repression of *STOMAGEN* gene expression by the TF MONOPTEROS (also known as ARF5) (Zhang et al., 2014). In the absence of light, stomatal differentiation is suppressed. This requires proper auxin biosynthesis and signaling, and involves AUXIN RESISTANT 3 (AXR3) (also known as IAA17) (Balcerowicz and Hoecker, 2014; Balcerowicz et al., 2014). Genetic analyses place AXR3 upstream of YDA, largely overlapping with the ER family, but independent of TMM.

CO_2 levels also regulate the upstream ligands that control stomatal development. High CO_2 concentration not only triggers stomatal closure but also reduces stomatal density in the long term (Casson and Gray, 2008). The *HIGH CARBON DIOXIDE* (*HIC*) gene, which encodes a biosynthetic enzyme for epicuticular wax, and two carbonic β-anhydrase genes, *CA1* and *CA4*, have been shown to play a role in high CO_2-mediated repression of stomatal development (Engineer et al., 2014; Gray et al., 2000). Interestingly, elevated CO_2 induces the expression of CO_2 RESPONSIVE SECRETED PROTEASE (CRSP), a subtilase that cleaves the EPF2 pro-peptide to generate mature, bioactive EPF2 (Fig. 3) (Engineer et al., 2014). In addition, elevated CO_2 upregulates the expression of *EPF2* itself, thereby constituting a feed-forward loop to increase the upstream inhibitory signal for stomatal differentiation (Fig. 3) (Engineer et al., 2014). CRSP possesses a cleavage activity specific to EPF2 and not to EPF1. This suggests that yet another subtilase may exist to control different steps of stomatal development.

Stem cell polarity and the asymmetric segregation of cell fate determinants

A stomatal meristemoid possesses intriguing characteristics that allow it to reiterate ACDs, each time renewing itself as well as giving rise to an SLGC. Each asymmetric amplifying division of the meristemoid occurs precisely at a 60° angle (Serna et al., 2002), leading to an inward spiral pattern that constitutes the characteristic anisocytic stomatal complex seen in *Brassicaceae*. Indeed, mathematical modeling predicts that a simple polarity switching mechanism can explain the patterns of asymmetric amplifying divisions (Robinson et al., 2011).

Prior to an ACD, the nucleus of a meristemoid migrates to the apical end while a vacuole localizes to the basal end; these then predicate meristemoid and SLGC fates, respectively (Nadeau and Sack, 2002a). Thus, intrinsic polarity within a meristemoid is tightly coupled with its binary cell fates. This inherent connection between cell polarity and cell fate is yet another example of shared conserved logic between plants and animals (Abrash and Bergmann, 2009). During *Caenorhabditis elegans* embryogenesis and *Drosophila melanogaster* neurogenesis, for instance, a group of evolutionarily conserved PAR proteins plays a key role in controlling asymmetric cell division. PAR proteins are initially localized uniformly. Upon symmetry breaking, they exhibit characteristic polar localization, defining posterior versus anterior (or apical versus basal) polarity, and directing the unequal segregation of cell fate determinants that specify the fates of two daughter cells (Gönczy, 2008). However, it should be noted that the actual mechanisms responsible for executing ACDs in plants and animals appear to differ. During cytokinesis in animal cells, an actomyosin-based contractile ring generates a physical force to separate the two daughter cells (Gönczy, 2008). Unlike animal cells, however, plant cells are encapsulated by cell walls and, as such, cytokinesis is achieved through *de novo* formation of a cell plate. The cell plate forms at the location predetermined by the preprophase band, initiates as a disc in the middle of the cell, and is built outwards guided by phragmoplasts (specialized microtubules along which vesicles carrying building materials are delivered) to manifest eventually as a new cell wall that fully separates the two daughter cells (Müller and Jürgens, 2015). These disparate processes of cytokinesis in plant and animal cells imply that ACDs and the segregation of cell fate determinants might also be executed by completely different regulators.

The intrinsic control of ACD and cell expansion by BASL and the MAPK cascade

The key intrinsic regulator of ACDs in meristemoids was identified from a mutant, *breaking of asymmetry in the stomatal lineage* (*basl*), in which a meristemoid occasionally fails to undergo ACD, giving rise to two meristemoids as daughter cells (Dong et al., 2009). BASL protein, which represents a novel stomatal lineage-specific protein of *Brassicaceae*, exhibits dramatic subcellular localization dynamics during ACD (Fig. 4). Using a GFP-BASL fusion protein, it was shown that BASL is localized in the nucleus in meristemoids. During ACD, a fraction of GFP-BASL migrates to the cell cortex at the distal end of the meristemoid, where cell expansion will proceed. After the ACD, GFP signals stay in the nucleus of the newly formed meristemoid, but remain at the cortex of the SLGC (Fig. 4A). When the SLGC undergoes an asymmetric spacing division, GFP-BASL reorients away from a new site of ACD. BASL protein then disappears as the meristemoid differentiates into a GMC (Fig. 4B) (Dong et al., 2009). Interestingly, ectopically overexpressed GFP-BASL in hypocotyl cells localizes to the basal end and causes cell bulging (Dong et al., 2009). This suggests that BASL marks the site of cell expansion and
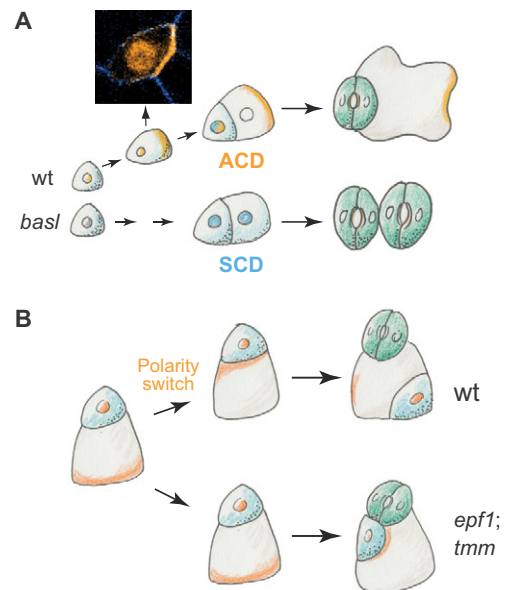

Fig. 4. The intrinsic and extrinsic control of polarity within the stomatal lineage. (A) In wild-type (wt) meristemoids (light cyan), BASL (orange) accumulates in the nucleus. Prior to asymmetric cell division (ACD), a fraction of BASL translocates to the cell cortex at the distal end of the cell. After the ACD, BASL stays in the nucleus of the meristemoid, which eventually expresses MUTE (cyan) and differentiates into a stomata. In the sister SLGC, BASL stays localized at the cell cortex, and this leads to high MAPK activity, loss of stomatal precursor state, and eventual differentiation to a pavement cell. In *basl* mutants, meristemoids occasionally produce two daughter cells of the same fate; this can be termed a symmetric cell division (SCD). Here, both daughter cells express MUTE (cyan) and hence differentiate into stomata. The confocal microscopy image shows a meristemoid preceding the ACD. BASL-GFP (orange) fusion protein is detected both in the nucleus and at the cell-cortex of the future SLGC. The confocal image was provided courtesy of Dr Juan Dong (Rutgers University, NJ, USA). (B) Extrinsic signals influence BASL dynamics. In wild-type plants, prior to an asymmetric spacing division, BASL protein (orange) switches its polar localization within the SLGC (gray), which re-acquires a meristemoid mother cell (MMC) fate. This ensures that the secondary meristemoid (cyan) forms at least one cell apart from the pre-existing stoma (one-cell spacing rule). In *epf1* or *tmm* mutants, this polarity switch is abrogated, resulting in mis-orientation of the spacing division and, consequently, stomatal clustering.

promotes the cell expansion process, probably by recruiting the regulatory components.

Consistent with this, a recent report deciphered that BASL functions as a scaffold for MAPK signaling components (Zhang et al., 2015). BASL directly associates with YDA and recruits YDA to the cell cortex in future SLGCs. BASL can be directly phosphorylated by MPK6 *in vitro*, and this phosphorylation is necessary for BASL polarity and hence proper ACDs *in vivo*. The emerging scenario is that positive feedback between BASL and the MAPK cascade specifies an ACD. Phosphorylated BASL exhibits polar localization, dragging the MAPK components to the cell cortex, where MAPK activities intensify (Zhang et al., 2015). The high MAPK activity is inherently coupled with cell expansion and repression of stomatal precursor fate by degrading SPCH and MUTE in the nucleus, thereby promoting SLGC/pavement cell fate (Wang et al., 2007). This way, BASL ensures that only one daughter cell of the meristemoid will retain stomatal precursor identity (Fig. 4A).

Like BASL, the novel coiled-coil protein POLAR and the closely related POLAR-LIKE1 exhibit dynamic asymmetric localization patterns during ACDs within the stomatal lineage (Adrian et al., 2015; Pillitteri et al., 2011). POLAR-GFP localizes uniformly in a meristemoid, but prior to ACD the POLAR-GFP signal distributes sharply to the cell cortex at the distal end of the meristemoid. This requires *BASL*, suggesting that POLAR acts downstream of the BASL machinery (Pillitteri et al., 2011). The exact biological functions of POLAR and its paralog remain unclear. It is worth mentioning that transcriptomic analyses of meristemoid-enriched populations, using FACS or a *scrm-D*-enabled background, identified a group of 'novel' genes that are highly specific to stomatal cell lineages (Adrian et al., 2015; Pillitteri et al., 2011). It would be fascinating to examine whether any of them encode novel polarity components.

The role of auxin in the control of ACDs within the stomatal lineage
Despite the identification of BASL and POLAR, and their key roles in regulating ACDs, a number of questions still remain. For example, what delivers the BASL-MAPK complex to the site of cell expansion? How does the BASL-MAPK module trigger polar cell expansion? Recent studies indicate that the plant hormone auxin might be involved in these processes. The asymmetric distribution of auxin plays a pivotal role in ACDs during embryogenesis and in root meristems (Casimiro et al., 2001; De Smet and Beeckman, 2011; Friml et al., 2003). Auxin activates small Rho-like GTPases (ROPs) (Tao et al., 2002; Yalovsky et al., 2008), which in turn regulate the polar distribution of PIN-FORMED (PIN) auxin efflux carriers (Lin et al., 2012). Although the involvement of ROPs in meristemoid ACDs remains unclear (Dong et al., 2009), it has been noted that proper auxin transport greatly influences stomatal patterning (Le et al., 2014). The auxin efflux carrier PIN3 is highly and transiently expressed in meristemoids, and time-lapse imaging shows that PIN3-GFP is maintained in the meristemoid after ACD whereas it disappears from its sister SLGC. By contrast, the auxin output marker DR5-VENUS is maintained in the SLGC whilst disappearing from the meristemoid. These observations suggest that the timely efflux of auxin from stomatal precursors is crucial for ACD. Consistently, higher-order *pin* mutants (e.g. *pin2/3/4/7*) as well as treatments with birferdin A (an inhibitor of recycling and endocytosis) or NPA (naphthylphthalamic acid; an auxin polar transport inhibitor) confer occasional symmetric divisions of meristemoids, leading to paired stomata resembling those seen in the *basl* phenotype (Le et al., 2014). The observed correlation of increased auxin and SLGC fate implies that the high auxin state stimulates cell expansion and inevitable differentiation

of SLGCs to pavement cells. Whether this involves any of the previously known components of auxin-mediated cell expansion is an open question.

Other signals controlling ACDs during stomatal development
Stem cell divisions, in both plants and animals, are also regulated by signals from the environment. Examples include *Drosophila* male germline stem cells, in which daughter cells require physical contact with the hub (a group of supporting cells) to maintain stemness (Inaba et al., 2015; Yamashita et al., 2005). During stomatal development, EPF1-ERL1/TMM peptide-receptor kinase signaling is required to orient the polarity of the asymmetric spacing divisions (Lee et al., 2012). These cell-cell signaling components act upstream of BASL, as GFP-BASL mislocalizes in SLGCs of *epf1* and *tmm* mutants (Dong et al., 2009). However, no clear asymmetric distribution of TMM or ERL1 has been reported. Moreover, both TMM and ERL1 show highest expression in meristemoids, which also express EPF1 (Hara et al., 2007; Nadeau and Sack, 2002b; Shpak et al., 2005). This raises the question of how these signaling components orient the site of ACD. It is possible that transient activation of the TMM-ERL1 receptor complex in SLGCs is sufficient to activate MAPKs, which in turn promote polar localization of BASL. The overexpression or application of bioactive EPF1 peptides results in arrested meristemoids/GMCs, resembling the *mute* phenotype (Hara et al., 2007; Lee et al., 2012). High accumulation of ERL1 and TMM in meristemoids might somehow buffer the endogenous EPF1 signals to prevent self-activation of the inhibitory signaling pathway.

The polar localization of receptor kinases during ACD is well documented in the case of stomatal development in maize. This grass species undergoes a characteristic cell division sequence, giving rise to a stomatal complex with dumbbell-shaped GCs. Instead of asymmetric amplifying divisions of meristemoids, asymmetric divisions of subsidiary mother cells (SMCs), which neighbor GMCs, generate subsidiary cells with specialized functions (Peterson et al., 2010; Stebbins and Shah, 1960). Two Maize LRR-RLKs, PANGROSS2 (PAN2) and PAN1, as well a ROP protein, promote the pre-mitotic polarity and subsequent ACD of SMCs. They exhibit polar localization along the plasma membrane of SMCs facing the GMC (Cartwright et al., 2009; Humphries et al., 2011; Zhang et al., 2012). The obvious and exciting scenario is that these signaling receptors perceive as yet unidentified positional cues, probably secreted peptides, from the neighboring GMC to orient ACDs. Genetic studies show that the polar localization of PAN2 precedes that of PAN1 (Zhang et al., 2012). Subsequently, receptor activation results in the recruitment of ROPs and other downstream components. The earliest SMC polarity markers, BRICKs, are components of the SCAR/WAVE regulatory complex, which in animals activates the actin nucleating ARP2/3 complex (Facette et al., 2015; Frank et al., 2003). Thus, a branched actin network might be a prerequisite for polar localization of PAN2 LRR-RLK. These unique players in grass SMC ACDs imply diversity in evolutionary co-opted division programs and their choices of players in regulating stomatal complex development in different plant taxa.

Conclusions and perspectives
Recent studies of stomatal development have started to unveil specific functions of master regulatory transcription factors in specifying the initiation, proliferation and differentiation of stomata. The consecutive use of bHLH heterodimers and their regulation by cell-cell signals bears similarities to the molecular mechanisms adopted by animals to generate specialized cell types. At the same time, the epigenetic

regulation of FAMA and FLP underscores the reprogrammable nature of the plant cell. Terminally differentiated GCs readily reprogram and re-initiate the stomatal stem cell fate unless the chromatin is maintained in a repressive state. The identification of key signal transduction components, secreted peptide ligands, receptor kinases, and MAPK components has opened the door to the possibility of dissecting signal specificity, interference and integration during stomatal development. Furthermore, the discovery of BASL and its association with MAPK components has unveiled a unique innovation used by plant cells to execute ACDs.

There are, however, important questions in stomatal development that are under-investigated, but recent technological advances are beginning to provide us with the means to address these questions. For example, the accessibility of the epidermis to long-term time-lapse analyses (Peterson and Torii, 2012), coupled with quantitative, high-resolution four-dimensional imaging, could elucidate the dynamics of signal perception, activation, and cell fate specification. Mathematical modeling (Horst et al., 2015; Robinson et al., 2011) could also be implemented to verify the circuits involved in stomatal development and predict missing components.

Finally, understanding the evolution of stomatal development may provide crucial insights into the evolution and adaptation of land plants. Owing to their essential physiological functions, the development of stomata is evolutionarily constrained in the land plants. Indeed, the master regulatory bHLHs are conserved down to non-vascular, basal land plants, such as moss (MacAlister and Bergmann, 2011; Peterson et al., 2010). However, the arrangements of stomata in the epidermis exhibit diversity among taxa (Peterson et al., 2010). It is entirely possible that each plant taxon has adopted unique signaling or polarity components to enforce the one-cell spacing rule. This is exemplified in the distinct molecular components driving the ACDs of SLGCs in *Arabidopsis* versus SMCs in maize. Very recent genome sequencing analysis of seagrass, a flowering plant that lives entirely under the sea, revealed that this species lost the suite of genes regulating stomatal development: indeed, *SPCH*, *MUTE*, *FAMA*, *SCRM2*, *FLP*, *MYB88*, *EPF1*, *EPF2*, *STOMAGEN* and *TMM* are absent from the seagrass genome (Olsen et al., 2016). This striking discovery emphasizes that loss of the stomatal developmental pathway signifies the adaptation of land plants to an aquatic environment. Further evo-devo approaches may unravel unique regulatory circuits underlying plant diversity and draw surprising parallels between the tissue patterning systems used in the plant and animal kingdoms.

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DEVELOPMENT