WUSCHEL is a primary target for transcriptional regulation by *SPLAYED* in dynamic control of stem cell fate in *Arabidopsis*

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SNF2 chromatin-remodeling ATPases play an important role in ensuring proper development in higher eukaryotes by controlling accessibility of *cis*-regulatory DNA regions to transcription factors and to the transcriptional machinery. However, the biological targets controlled by these ATPases are largely unknown. Using genetic and molecular analyses we have identified *WUSCHEL* (*WUS*) as a biologically important target of the SNF2-class ATPase *SPLAYED* (*SYD*) in the shoot apical meristem of *Arabidopsis*. We present evidence that *SYD* is recruited to the *WUS* promoter and that it is involved in regulation of the stem cell pool maintenance via direct transcriptional control of this master regulator.

[**Keywords:** Chromatin-remodeling ATPase; shoot apical meristem; SWI/SNF; stem cell fate; epigenetic regulation; *WUSCHEL*]

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Developmentally regulated gene expression is known to be controlled by evolutionarily conserved SWI/SNF ATPase complexes. Loss-of-function mutations in the central SNF2 ATPase subunits lead to a variety of developmental abnormalities (Tsukiyama 2002). Evidence is accumulating that SNF2 ATPases play important roles in cell division, differentiation, and embryo patterning in higher eukaryotes (Kennison and Tamkun 1988; Reyes et al. 1998; Bultman et al. 2000; Sawa et al. 2000; Wagner and Meyerowitz 2002; Gregg et al. 2003; Farrona et al. 2004).

Transcriptional regulation of many genes is dependent on SNF2-containing chromatin-remodeling complexes (Peterson and Workman 2000; Martens and Winston 2003), which control the access of *trans*-acting transcriptional regulators or of components of the general transcriptional machinery to the packaged eukaryotic genome (Emerson 2002; Narlikar et al. 2002). These complexes are recruited to *cis*-regulatory DNA regions by sequence-specific transcriptional activators/repressors or by specific histone modifications present at these sites (Peterson and Workman 2000; Martens and Winston

2003). This recruitment results in ATP-dependent alteration of the nucleosome position on the DNA and/or weakening of the DNA–histone interaction (Becker and Horz 2002; Lusser and Kadonaga 2003).

A given developmental defect observed in loss-of-function mutants for individual SNF2 chromatin-remodeling ATPases could thus be caused by (1) the critical perturbation of the expression of a master regulator gene or (2) the summation of effects on expression of multiple target genes involved in the developmental process under investigation. Available experimental evidence aimed at identifying molecular targets of a chromatin-remodeling ATPase in other organisms did not distinguish between these possibilities (Holstege et al. 1998; Sudarsanam et al. 2000; Armstrong et al. 2002; Damelin et al. 2002; Ng et al. 2002). For instance, whole-genome analyses performed in yeast show that expression of 6% of all genes is dependent on the SWI2/SNF2 ATPase (Holstege et al. 1998; Sudarsanam et al. 2000), suggesting that expression of a substantial number of genes can be affected by the absence of a chromatin-remodeling ATPase. However, only a subset of these genes are expected to be direct targets. Moreover, it is not certain if all direct target genes are biologically important in the developmental process under investigation.

The *Arabidopsis* proteome contains 42 ATPases that have the seven diagnostic SWI2/SNF2 motifs (<http://www.chromdb.org>). Phylogenetic analyses indicate that

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the *Arabidopsis* ATPases include representatives of all the conserved chromatin-remodeling subfamilies: SNF2, ISWI, CHD, DDM1, and SRCAP (Verbsky and Richards 2001; <http://www.chromdb.org>). Chromatin remodeling has been demonstrated for the *Arabidopsis* ATPase DDM1, suggesting that the *Arabidopsis* ATPases are functionally conserved with their yeast and/or mammalian counterparts (Brzeski and Jerzmanowski 2003). Four *Arabidopsis* genes are classified as members of the SNF2 subfamily of transcriptional coregulators (Verbsky and Richards 2001). In contrast, metazoans usually have one or two SNF2 genes, suggesting that the *Arabidopsis* ATPases may have more specific roles than their metazoan counterparts. In support of this idea, mutations in the single SNF2 ATPase in *Drosophila* cause lethality (Kennison and Tamkun 1988), while mutants of the *Arabidopsis* SNF2 ATPases SYD (Wagner and Meyerowitz 2002) and AtBRM (Farrona et al. 2004) are viable.

syd mutants have a variety of developmental defects, including short stature, slow growth, leaf polarity defects, ovule growth arrest, and loss of maintenance of the shoot apical meristem (SAM) (Wagner and Meyerowitz 2002; Eshed et al. 2004). This phenotypic pleiotropy is consistent with the idea that SNF2 chromatin-remodeling ATPases regulate transcription of genes involved in many developmental processes (Peterson and Workman 2000; Emerson 2002; Martens and Winston 2003). We were particularly interested in studying the basis for the shoot apical meristem defect in *syd* because a phenotypic defect is observed in the *syd* single mutant and because the signaling pathway that regulates SAM maintenance in plants is genetically well defined. This provides an opportunity to identify the specific targets of SYD regulation in SAM maintenance and to investigate whether the developmental defect in *syd* is the result of a defect in transcriptional regulation of a critical master gene such as *SHOOT MERISTEMLESS* (*STM*) and *WUSCHEL* (*WUS*), or of transcriptional misregulation of multiple SAM regulators.

Here we provide genetic and molecular evidence that SYD, a SNF2 class ATPase, plays a role in regulation of the stem cell pool in the SAM primarily via direct transcriptional control of the master SAM regulator *WUS*.

Results

SAM defects observed in the syd-2-null mutant plants

Adult *syd* plants undergo premature termination of the SAM; carpelloid structures are formed after the inflorescence SAM has given rise to two to 20 flowers (Fig. 1C; Wagner and Meyerowitz 2002). In contrast, the wild type maintains the SAM until it senesces. In order to investigate when the SAM defect in the *syd* plants is first detectable, we compared SAM development in the *syd-2*-null mutant with that in the *Ler* wild type at various developmental stages using scanning electron microscopy (SEM) (Fig. 1A). During the vegetative stage (at days 5 and 12), there were no discernable differences in SAM morphology between *Ler* and *syd-2* plants. However, a

Figure 1. Morphological and molecular differences in the shoot apical meristem (SAM) between *Ler* and *syd-2* plants. (A) Scanning electron microscope (SEM) images of the SAM in the wild-type *Ler* and *syd-2* at different developmental stages. Days after germination are indicated. The SAM at day 19 is marked by white asterisks and stage 4 flowers are indicated (4). Bars, 100 μm. (B) Semiquantitative RT-PCR analysis of known meristem regulators. Three independent RNA isolations each of wild-type (*Ler*) and *syd-2* mutant seedlings were compared. RNA was isolated from the entire above-ground plant tissues 18 d after germination. (C) Photographs of *syd-2* plants after 2 mo of growth show carpelloid structures produced after SAM termination while *Ler* plants retain their SAM until they senesce (data not shown). Bar, 1 mm. (D) The width and height of the SAM in *Ler* and *syd-2* plants at day 21 after germination were measured in histological sections. More than seven SAMs were used for measurements, and the standard error of the mean is shown. (E) Quantification of message levels in B. The mean intensity of three independent RT-PCR products in *syd-2* was set to 1. The standard error of mean is shown. The genes abbreviated are *CLAVATA* (*CLV*), *POLTERGEIST* (*POL*), *WUSCHEL* (*WUS*), *SHOOT MERISTEMLESS* (*STM*), *ASYMMETRIC LEAVES* (*AS*), *KNOTTED-like from ARABIDOPSIS THALIANA* (*KNAT*), and *Eukaryotic Translation Initiation Factor 4A-1* (*EIF4A*).

reduction in SAM size was evident in *syd-2* during early reproductive development (day 19), and became even more pronounced at later stages of inflorescence development (day 40). The number of visible flower primordia at stage 4 or younger was reduced in *syd-2* compared

with *Ler* at day 19, suggesting a possible defect in SAM activity (Fig. 1A). Consistent with this, the width of the SAM was reduced in *syd-2* plants at an early reproductive stage (day 21) (Fig. 1D). At day 54, the SAM in *syd-2* plants was consumed, while *Ler* plants retained their SAM. Defects in the SAM are thus mainly apparent during the reproductive stage in *syd-2*-null mutants.

SAM maintenance is regulated primarily through two independent pathways by the homeodomain transcription factors WUS and STM (Clark 2001; Baurle and Laux 2003; Carles and Fletcher 2003). In one pathway, the size of the stem cell pool in the SAM is dynamically regulated by a negative feedback loop involving WUS and CLAVATA (CLV) (Brand et al. 2000; Schoof et al. 2000). WUS confers stem cell fate on the overlying cell layers in an unknown non-cell-autonomous manner (Laux et al. 1996; Mayer et al. 1998). The stem cells thus specified express the CLV3 protein ligand resulting in negative regulation of WUS through the CLV1/CLV2 leucine-rich repeat receptor kinase complex (Fletcher et al. 1999; Jeong et al. 1999; Trotochaud et al. 1999; Rojo et al. 2002; Lenhard and Laux 2003). The KNOX-class homeodomain transcription factor STM regulates a parallel pathway controlling the initiation and maintenance of the SAM (Long et al. 1996). STM maintains the SAM by preventing premature differentiation of meristem cells (Barton and Poethig 1993; Endrizzi et al. 1996). It does so by restricting the expression of *ASYMMETRIC LEAVES1* (*AS1*) and *ASYMMETRIC LEAVES2* (*AS2*) genes to organ primordia, where these genes are required for differentiation and for proper leaf development (Byrne et al. 2000; Tsiantis and Hay 2003). *AS1* and *AS2* negatively regulate the expression of KNOX genes, such as *KNAT1* and *KNAT2*, which can compensate for the loss of STM in the absence of *AS1* and *AS2* (Byrne et al. 2000; Tsiantis and Hay 2003).

Since SYD, as a SNF2-class ATPase, is predicted to play a role in transcriptional regulation (Narlikar et al. 2002; Martens and Winston 2003), we compared the expression levels of known meristem regulators in 18-d-old wild-type (*Ler*) and *syd-2* plants (Fig. 1B,E). At the stage during reproductive development when phenotypic defects in the SAM were first observed (Fig. 1A,D), the expression of most meristem regulators was not significantly altered (Fig. 1B,E). However, we detected a considerable reduction in the message levels of *WUS* and *CLV3* in *syd-2* relative to wild type. Consistent with the absence of a morphological difference between wild-type and mutant vegetative meristems, *WUS* and *CLV3* expression was not reduced during the vegetative stage in *syd* mutants (data not shown; see Fig. 5A, below). Thus, the SAM defect in *syd-2* during the reproductive stage is correlated with a reduction of *WUS* and of *CLV3*.

Genetic interaction between SYD and meristem-regulating transcription factors

To further test whether SYD regulates meristem maintenance via the WUS/CLV3 pathway rather than the STM pathway, we constructed the double mutants

wus-1 syd-2, *stm-2 syd-2*, and *stm-1 syd-2*. Double mutants between *wus-1* and weak *stm* alleles are known to result in strong meristem defects (Endrizzi et al. 1996) presumably because of the cooperative action of WUS and STM on SAM maintenance (Gallois et al. 2002; Lenhard et al. 2002).

If SYD acts in the STM pathway, we expect a similar phenotype in *wus-1 syd-2* to that observed in the double mutant between *wus-1* and a weak *stm* allele. In *wus-1* plants, two young primary leaves were visible at day 14 (Fig. 2A,Q), and many irregular-shaped leaves (Fig. 2C) were formed from an abnormally flat meristem at later stages (Fig. 2D) as previously reported (Laux et al. 1996; Mayer et al. 1998). In contrast, no primary leaves were visible in *wus-1 syd-2* plants at day 14 (Fig. 2E,R), suggesting a possible enhancement of *wus-1* by *syd-2*. However, cotyledon petiole fusion was absent in *wus-1 syd-2* in contrast to the strong petiole fusions observed in double mutants between *wus-1* and a weak *stm* allele (Fig. 2R,S; Endrizzi et al. 1996), indicating that the phenotypic difference between *wus-1* and *wus-1 syd-2* is not derived from reduced STM function. Furthermore, closer observation by SEM analyses revealed the presence of a small, undifferentiated cell population (Fig. 2F) between the cotyledon petioles at this stage (day 12), suggesting presence of an active meristem in the double mutant. Consistent with this, small and partially radialized leaves developed in half of *wus-1 syd-2* plants by day 28 (Fig. 2G,H; data not shown). This defect is consistent with a role of SYD in leaf polarity regulation (Eshed et al.

Figure 2. Genetic interactions between SYD and two homeodomain meristem regulators. Pictures of green plants and cleared seedlings were taken 14 d after germination except for C and G (28 d after germination). SEM images of the SAM were from 12-d-old plants (B,F,J,N) or 26-d-old plants (D,H,L,P). Shoot meristems are indicated with white asterisks. The two primary leaves produced in *wus-1* (D14) are indicated by an arrowhead. Bars: in the green plant pictures, 2.5 mm; G, 1 mm; in the SEM images, 100 μ m. Genotypes of green plants are indicated in the corresponding SEM images to the right.

2004) and may contribute to the delayed leaf emergence and/or the reduced leaf size in *wus-1 syd-2*. Presence of residual meristem activity in *wus-1 syd-2* as well as absence of cotyledon fusion in this double mutant together support the idea that SYD is not required for the STM SAM-maintenance pathway.

If SYD acts primarily in the WUS pathway, we expect a significant enhancement of a weak *stm* mutant by *syd-2* (Endrizzi et al. 1996). Strong *stm* alleles exhibit near complete loss of SAM function and thus may only be marginally enhanced. In the weak *stm-2* mutant a shoot meristem is initiated but arrested after making a few leaves (Fig. 2K), followed by repetitive initiation of defective shoot meristems (Fig. 2L), eventually resulting in a bushy adult plant (Clark et al. 1996). In contrast, *stm-2 syd-2* never formed primary leaves (Fig. 2O,P; data not shown), indicating that *syd-2* strongly enhances *stm-2*. When we combined *syd-2* with the stronger *stm-1* mutant, no primary leaves were visible at day 14 in either *stm-1*, as previously reported (Barton and Poethig 1993), or in *stm-1 syd-2* double mutants (Fig. 2I,J,M,N). While some *stm-1* plants showed partial rescue of the meristem defect later in development (Clark et al. 1996), no phenotypic rescue could be detected in *stm-1 syd-2* plants (data not shown), suggesting that *stm-1 syd-2* plants have a more severe defect in meristem function than *stm-1* plants. The combined genetic data are consistent with SYD acting primarily in the WUS pathway, as was suggested by the reduction of *WUS* and *CLV3* in *syd-2* compared with wild type (above).

SYD is required for WUS transcription

To verify the observed genetic interactions, we assayed gene expression levels in the single versus double mutants to evaluate the potential effects of *syd* on expression of *WUS/CLV3* and *STM*. In *stm-2 syd-2*, a 12-fold reduction in *WUS* was observed compared with *stm-2*, while a lesser, but obvious, reduction occurred in *CLV3* and in *STM* (Fig. 3A,B). The pronounced reduction of *WUS* (Fig. 3A,B) is likely the primary cause for the enhanced SAM defect in *stm-2 syd-2*. Because *WUS* is a positive regulator upstream of *CLV3*, the reduction in *CLV3* could result from the reduced *WUS* activity. The observed reduction of *STM*, on the other hand, is likely due to the reduced meristem size of *stm-2 syd-2* (Fig. 2O,P). We conclude that SYD primarily regulates *WUS* expression.

Because of their near complete loss of SAM activity, *stm-1* mutants have very low levels of *WUS* compared with *wus-1* or *stm-2* (Fig. 3A). While *WUS* expression was further reduced in *stm-1 syd-2*, this reduction was only twofold (Fig. 3A,B) possibly due to the already very low *WUS* expression in *stm-1*. The observed reduction in *STM* and *CLV3* (Fig. 3A,B) is consistent with an enhancement of the SAM defect of *stm-1* by *syd-2*.

In *wus-1 syd-2*, we observed a very strong reduction in *WUS* message. Note that the *wus-1* mutation (Mayer et al. 1998) causes a splicing defect (see Materials and

Figure 3. Meristem-regulator expression in *syd* mutant meristems. (A) RT-PCR was performed using total RNA isolated from above-ground tissues of vegetative 12-d-old seedlings. In *stm-2* plants, a mature and expanded leaf was removed before RNA isolation because all other plants did not have mature leaves at this stage. The point mutation in *wus-1* causes the removal of the second exon resulting in a smaller transcript. (B) Quantification of message levels. Intensities of the respective RT-PCR products from A were normalized by the intensities of *EIF4A*. The fold change was obtained by dividing the signal strength in the single mutant by that of the double mutant. Mutant messages are indicated by black asterisks. (C) GUS analysis of *WUS* and *CLV3* in 12-d-old wild-type and mutant plants. Strong GUS signals were detected in *Ler* and *syd-2* plants after prolonged incubation (16 h) at 37°C. The insets show a magnified view of the GUS-expressing cell population in *stm-2* seedlings. The arrowhead indicates the GUS-expressing cell population. Bar, 1 mm.

Methods), which results in a smaller transcript (Fig. 3A). This smaller transcript is expected to give rise to a truncated protein consisting of the N-terminal 144 amino acids of *WUS*. The presumptive truncated protein includes the entire homeodomain. We also observed a slight reduction in *CLV3* and *STM* message in *wus-1 syd-2* compared with *wus-1* (Fig. 3A,B). The marginal reduction in *CLV3* and *STM* expression could suggest that SYD, in addition to regulating *WUS*, may play a minor role in other SAM-regulating pathways. Alternatively, if *wus-1* is not a true null allele, the strong reduction of *WUS-1* message by *syd-2* could result in an additional reduction of SAM function (and hence *CLV3* and *STM* expression) in this double mutant.

The reduction in *WUS* and *CLV3* transcript levels in *stm-2 syd-2* compared with *stm-2* was confirmed by promoter activity analysis using GUS reporters. Both *WUS*:GUS and *CLV3*:GUS were detected in *stm-2*, whereas no GUS activity was detected in *stm-2 syd-2* plants even after prolonged incubation with the substrate (Fig. 3C). We conclude that SYD regulates maintenance of the stem cell pool primarily via control of *WUS* expression.

SYD is required for cotyledon separation

We noted a second phenotype in *stm syd* double mutants. These mutants exhibited strong cotyledon fusion (Fig. 2S–V). The fusion in *stm-2 syd-2* exceeded that observed in *stm-1*, one of the strongest *stm* alleles (Fig. 2, cf. S and V; Barton and Poethig 1993). When we combined *syd-2* with *stm-1*, enhanced cotyledon petiole fusion was observed compared with *stm-1* (Fig. 2, cf. S and T). Thus, in addition to SAM maintenance, SYD regulates cotyledon fusion. It is known that *STM* and the *CUP-SHAPED COTYLEDON* (*CUC*) genes can affect cotyledon separation (Aida et al. 1997, 1999; Takada et al. 2001; Vroemen et al. 2003). Because *syd* strongly enhances the cotyledon fusion defect in *stm-1* and because SYD does not appear to act upstream of *STM*, SYD is not likely to control cotyledon separation via *STM*. We therefore monitored the expression of the *CUC* genes (*CUC1*, *CUC2*, and *CUC3*), which are implicated in cotyledon separation and organ boundary formation (Aida et al. 1997; Takada et al. 2001; Vroemen et al. 2003), in the double mutants. SYD regulation of these genes may be correlated with the enhanced cotyledon fusion we observed in *stm-2 syd-2* and *stm-1 syd-2* plants (Fig. 2T,V). Indeed, we observed a strong reduction in the message abundance of *CUC2* and *CUC3* but not of *CUC1* in these mutants, suggesting a selective requirement for SYD in *CUC* expression (Fig. 3A,B). Absence of either *CUC1* or *CUC2* function in the background of *stm* mutants is known to enhance cotyledon fusion (Aida et al. 1999). Therefore, the reduction of transcript levels in *CUC2* and *CUC3* in *stm-2 syd-2* and *stm-1 syd-2* (Fig. 3A,B) is likely the main cause for the strong enhancement of cotyledon fusion in these mutants (Fig. 2T,V). The reduction of *CUC2* and *CUC3* messages is not likely to contribute to the SAM defect of *syd* mutants because *CUC* genes affect SAM function through *STM* (Takada et al. 2001), while SYD does not appear to regulate *STM* (see above).

Taken together our findings support the conclusion that SYD acts upstream of *WUS* in the regulation of stem cell fate. In addition, we showed that SYD plays a separate role in cotyledon separation via regulation of *CUC2* and *CUC3* but not *CUC1* expression.

clv1-4 partially rescues the SAM defect of syd-2

The *CLV1* receptor regulates *WUS* levels via a negative feedback loop. In *clv1* mutant plants, increased *WUS* transcription results in increased accumulation of stem cells and a subsequent increase in the size of the SAM (Clark et al. 1993, 1997; Fletcher et al. 1999; Brand et al. 2000; Schoof et al. 2000). The specificity of the effect of *CLV1* on *WUS* and the opposite phenotypes of *clv1* and *syd* mutants allowed us to further investigate whether SYD acts on *WUS* via *CLV* and the negative feedback loop.

We constructed double mutants between *syd-2* and *clv1-4*, a very strong dominant-negative allele (Dievart et al. 2003), to investigate the genetic interaction of these

proteins in SAM function (Fig. 4). The overall plant morphology of *clv1-4 syd-2* was similar to that of *syd-2* rather than that of *clv1-4*, indicating that other roles of SYD (e.g., in leaf and flower patterning) were not affected by *clv1-4* (Fig. 4A). However, we observed a striking difference in SAM morphology between *clv1-4* and *clv1-4 syd-2* plants during the vegetative stage. The SAM was enlarged in *clv1-4* seedlings but not in *clv1-4 syd-2* or wild-type seedlings (Figs. 4C, 1A). Later in development, overproliferation of the SAM was observed in *clv1-4* and *clv1-4 syd-2* (Fig. 4C, day 26). However, *clv1-4 syd-2* meristems tended to fasciate rather than to expand radially

Figure 4. Phenotypic comparison between *clv1-4* and *clv1-4 syd-2*. (A) Photographs of green plants taken 14 d after germination. (B) Leaf initiation rate (graph a) and total flower number produced (graphs b,c) under long day (graphs a,c) and continuous light (graph b) are shown. (Graph a) Leaf initiation rate is calculated as the number of new leaves produced per day between the fourth and the seventh true leaf. (Graphs b,c) Total flower number refers to flowers stage 12 and above. Black asterisks in B indicate termination of the SAM. (C) SEM images of the SAMs in single versus double mutants. Developmental time points are indicated. White asterisks in C indicate the SAM during vegetative and reproductive stages. Bars: A, 2.5 mm; C, 100 μ m.

like *clv1-4* meristems. This linear expansion may be viewed as a less pronounced SAM overproliferation (Clark et al. 1993, 1996). A large number of flower primordia initiated from the linearly enlarged SAM in *clv1-4 syd-2*, suggesting at least partial rescue of the SAM defect of *syd-2* by *clv1-4* (cf. Figs. 1A and 4C).

We next measured leaf initiation rate and counted total flower number (Fig. 4B) in an attempt to investigate SAM activity in wild-type and mutant plants. Leaf initiation rate and total flower number were increased in *clv1-4* compared with wild type in agreement with the increased SAM size in the mutant. The leaf initiation rate and total flower number of *clv1-4 syd-2* was markedly lower than that of *clv1-4* but higher than that of *syd-2* (Fig. 4B), indicating that the SAM activity of *clv1-4 syd-2* is intermediate between *clv1-4* and *syd-2*. The intermediate phenotype in *clv1-4 syd-2* indicates that *SYD* does not regulate *WUS* levels via inhibition of the *CLV* negative feedback loop.

It should also be noted that *clv1-4* rescues the SAM termination of *syd-2* plants during the reproductive phase (Fig. 4B, graphs b,c). Thus the reduced *WUS* activity in *syd-2* may be an important cause for the SAM maintenance defect in the mutant.

SYD is a limiting factor for elevated *WUS* transcription in *clv1-4*

CLV1 is a negative regulator of *WUS* and of the stem cell pool, a collection of undifferentiated cells that express *CLV3*. To ascertain whether the antagonistic interaction between *clv1-4* and *syd-2* results from opposite effects on *WUS* transcription, we investigated the message levels of *WUS*, *CLV3*, and *STM* in the mutants (Fig. 5A,B). In *clv1-4* plants, the *WUS* message is strongly increased compared with that of the wild type, consistent with the inhibitory role of *CLV* on *WUS* transcription. However, this increase in *WUS* expression was sharply reduced in the absence of *SYD* (Fig. 5A), indicating that *SYD* is a limiting factor for elevated *WUS* transcription in *clv1-4*. The reduction of *WUS* message levels in *clv1-4 syd-2* was more pronounced than that of all other genes tested (Fig. 5B), suggesting that *SYD* primarily affects *WUS* expression. Consistent with the role of *WUS* as an inducer of *CLV3* (Fletcher et al. 1999; Brand et al. 2000, 2002; Schoof et al. 2000), *CLV3* expression is reduced in *clv1-4 syd-2* compared with *clv1-4*; however, this reduction is much less pronounced than that of *WUS* (Fig. 5A,B). We also compared *WUS* expression in *clv1-4* versus *clv1-4 syd-2* using the *WUS::GUS* transgene as a reporter. We observed a strong reporter signal in a broad domain within the enlarged SAM of *clv1-4* seedlings (indicated by arrowheads in the light micrographs of a cleared seedling and of a histological section in Fig. 5C). In contrast, a weak *WUS::GUS* signal was detected in the center of the comparatively small SAM of *clv1-4 syd-2* plants (indicated by an arrowhead in the light micrograph of a cleared seedling and of a histological section in Fig. 5C). Both the size of the *WUS*-expressing domain as well as staining intensity appear to be reduced in *clv1-4 syd-2* compared with *clv1-4* (Fig.

Figure 5. *SYD* is a limiting factor for *WUS* transcription in *clv1-4*. (A) RT-PCR was performed to investigate the message abundance of *WUS*, *CLV3*, and *STM*. (B) The band intensities of the respective messages from A were normalized to the band intensities of *EIF4A*, and the normalized band intensities in *Ler* were set to one arbitrary unit. (C) GUS assays were performed in 8-d-old *clv1-4* and *clv1-4 syd-2* seedlings harboring a GUS transgene driven by the *WUS* promoter. Incubation with substrate was performed for 90 min at 37°C. GUS signals are shown from cleared plant seedlings (left photographs) and sectioned plants (right photographs). Bars, 100 μ m.

5C), suggesting that the pool of *WUS*-expressing cells as well as the level of *WUS* expression per cell is reduced in *clv1-4 syd-2*.

In summary, the reduction in SAM size and activity in *clv1-4 syd-2* versus *clv1-4* is correlated with strongly reduced levels of *WUS* transcript in the double mutant, consistent with the proposed role (above) for *SYD* in stem cell maintenance via regulation of *WUS* transcript levels.

SYD is recruited to the promoter region of *WUS*

Dependence of *WUS* message levels on *SYD* in a variety of SAM-mutant backgrounds and the molecular identity of *SYD* as a SNF2 ATPase suggest that *SYD* may act

directly on *WUS* transcription as a chromatin-remodeling factor through interaction with sequence-specific transcriptional activators. This hypothesis can be tested by chromatin immunoprecipitation (ChIP) assays, which monitor in vivo binding of regulatory factors to nucleosomal DNA (Orlando 2000). This method relies on immunoprecipitation of a protein bound (and cross-linked) to DNA, followed by PCR amplification of the precipitated DNA to test for enrichment compared with control immunoprecipitations using preimmune serum and relative to nonprecipitated (input) genomic DNA. Indeed, significant enrichment of genomic DNA harboring a defined *cis* element has been observed for ATP-dependent chromatin-remodeling factors in yeast and mammalian cell cultures using this technique (Orlando 2000; Damelein et al. 2002; Ng et al. 2002; Kadam and Emerson 2003). However, *WUS* is expressed in <10% of the cells of the SAM and <1% of the cells in young seedlings (Fig. 1; Mayer et al. 1998). In order to test whether SYD can be specifically recruited to the genomic *WUS* region for transcriptional activation, we therefore chose to use inflorescence tips of *clv1-4* because of the enlarged domain of *WUS*-expressing cells in this genotype and because *WUS* expression in this background is dependent on SYD (Figs. 4, 5).

For the ChIP we used a SYD-specific polyclonal antibody generated against the N-terminal domain (upstream of the ATPase) of SYD (Fig. 6B; Y. Su and D. Wagner, unpubl.). This antibody detects a prominent band in nuclear extracts of wild-type inflorescences, but not in those of *syd-2* mutants (Fig. 6B). We used a commercially available antibody against RNA polymerase II (RPO) to ensure equal loading of nuclear extracts from both wild-type and mutant inflorescences in this experiment (Fig. 6B).

Compared with the input and the preimmune serum control, the SYD antibody ChIP enriched the proximal *WUS* promoter region (site P) but only weakly precipitated the transcribed region of *WUS* (site G) in agreement with the presumed role of SYD as a coactivator of transcription (Fig. 6A). Relative to the input, the genomic regions further upstream of the *WUS* coding region were less strongly enriched (Fig. 6A, site D), suggesting that SYD preferentially associates with *WUS* promoter-proximal elements. This is consistent with the preferential association of SWI2/SNF2 ATPase with promoters rather than coding regions in target genes (Ng et al. 2002). In contrast, the SYD antibody ChIP did not enrich *STM* promoter-proximal (Fig. 6B, site P) or promoter-distal regions (Fig. 6B, site D) compared with the coding region (Fig. 6B, site G). These data suggest that SYD is specifically recruited to the *WUS* promoter.

As a control for protein occupancy at the sites tested and for ability to immunoprecipitate the two nucleosomal DNA templates, we next assayed for the presence of RNA polymerase II (RPO) at the *WUS* and the *STM* promoters. The proximal promoter regions (site P) of *WUS* and *STM* as well as the transcribed regions (site G) were both similarly enriched in ChIP experiments with the RPO antibody (Fig. 6), consistent with the role of

Figure 6. Recruitment of SYD to the *WUS* promoter. (A) Chromatin immunoprecipitation (ChIP) was performed with antibodies against SYD, RNA polymerase II (RPO), and LEAFY (LFY). Genomic regions amplified are indicated on the left. (D) Distal region; (P) proximal promoter region; (G) gene/coding regions. Precipitations were performed with preimmune serum (Preim.), SYD-specific antiserum (SYD), anti-RNA polymerase II antibodies (RPO), and anti-LEAFY antibodies (LFY). Genomic DNA (0.1%) was also amplified with each primer set (Input). (B, top) Relative ChIP enrichment represents the normalized ratios of the precipitated genomic DNA compared with 0.1% nonprecipitated genomic DNA (Input) after first subtracting the value of nonspecific precipitation by preimmune serum. The value of ChIP enrichment in the coding region (G) was set to 1 for both *WUS* and *STM*. SYD-specific antiserum (SYD) (left panel) and anti-RNA polymerase II antibodies (RPO) (right panel) were used for precipitation. (B, bottom left) Schematic diagram of the *WUS* and *STM* locus spanning promoter and coding regions. The *WUS* and *STM* open reading frame is shown by black boxes and the translation initiation site is indicated (arrow). The DNA regions amplified by PCR are shown above the genomic structure. (B, bottom right) Western analysis of nuclear extracts from wild-type and *syd-2* inflorescences probed with SYD (top panel) and RPO antibodies (bottom panel). SYD protein is detected strongly in wild type but absent in the *syd-2*-null mutant.

RNA polymerase in transcriptional initiation and elongation. This supports the interpretation that SYD preferentially binds to the proximal promoter region of *WUS*. To further examine the specificity of the SYD recruitment, we assayed for SYD binding to the promoter region of the eukaryotic translation initiation factor 4A (*EIF4A*). *EIF4A* is ubiquitously expressed, and its mes-

sage abundance is not dependent on SYD (Figs. 1, 3, 5). Hence *EIF4A* is not expected to be bound by SYD (Fig. 5). Relative to the input, we detected very weak association of SYD with the proximal promoter region of *EIF4A* (Fig. 6, EIF4P). Consistent with the ubiquitous expression of *EIF4A*, we detected very strong binding of RPO to this promoter (Fig. 6, EIF4P). Thus, SYD does not nonspecifically associate with promoters of transcriptionally active genes.

We noted considerable basal enrichment throughout the genomic regions tested in immunoprecipitation using SYD and RPO antibodies compared with those using preimmune serum (Fig. 6A), which is possibly due to an inherent nonspecific affinity of SYD and RNA polymerase to nucleosomal DNA. The RSC SWI2/SNF2 complex in yeast, for example, interacts nonspecifically with DNA and nucleosomes in vitro (Lorch et al. 1999; Sen Gupta et al. 2001). Consistent with this interpretation, the amount of DNA immunoprecipitated with an antibody against the LEAFY transcription factor (William et al. 2004) was comparable to that of the preimmune ChIP at most genomic sites (Fig. 6A).

We conclude that SYD is specifically recruited to the proximal promoter region of *WUS* in the SAM of *clv1-4* inflorescences. This indicates that the transcriptional regulation of *WUS* by SYD, which is important for control of stem cell fate, is direct.

Discussion

Many studies have focused on identifying molecular targets of a chromatin-remodeling ATPase (Holstege et al. 1998; Sudarsanam et al. 2000; Armstrong et al. 2002; Damelin et al. 2002; Ng et al. 2002). However, genetic analyses are required for identification of the biologically relevant target(s) and hence to understand the role of chromatin-remodeling ATPases in development. Using genetic and molecular analyses, we show here that *WUS*, a central regulator of stem cell fate, is a biologically relevant, direct target of SYD for SAM maintenance. This makes *WUS* the first direct target of a chromatin-remodeling ATPase in the plant kingdom. Furthermore, our results suggest that a specific developmental defect resulting from the loss of activity of a chromatin-remodeling ATPase can largely be attributed to perturbation of the expression of a single master regulator.

Here we investigated how SYD regulates SAM maintenance. We show that the meristem maintenance defect of the *syd* phenotype is not the result of expression perturbation of multiple target genes as a consequence of loss of SYD function. Instead, *WUS*, a central regulator of SAM maintenance, was found to be a functionally relevant target of SYD in the control of stem cell fate in the SAM. SYD acts in the *WUS* pathway because the SAM defect in *wus* was not substantially enhanced by *syd*, indicating that loss of SYD activity does not strongly affect meristem regulators in other meristem-regulating pathways. In support of this, we also observed a strong reduction of *WUS* transcript in *syd-2* single mutants during the reproductive phase and in several *syd*-

2-based double mutants. Furthermore, chromatin immunoprecipitation experiments indicate that SYD is specifically recruited to the proximal promoter region of the *WUS* locus, consistent with the genetic data and with the identity of SYD as a putative chromatin-remodeling factor. The antagonistic effects of CLV1 and SYD on SAM function indicate that SYD positively regulates the stem cell pool. In addition, the intermediate SAM phenotype of *clv1-4 syd-2* compared with each single mutant suggests that SYD does not regulate *WUS* via inhibition of the CLV pathway.

We do not exclude the possibility that SYD may have additional, minor, roles on expression of other meristem regulators such as *CLV3*. In fact, the expression domain of SYD in the SAM encompasses the expression region of *CLV3* and *STM* (Wagner and Meyerowitz 2002). The slight reduction of *CLV3* message levels in *wus-1 syd-2* compared with *wus-1* suggests that SYD may regulate *CLV3* expression directly or through an unknown *WUS*-redundant stem-cell-promoting pathway. However, any involvement of SYD in transcriptional regulation of these other potential targets is unlikely to contribute substantially to the SAM maintenance defect resulting from loss of SYD activity because the single- and double-mutant phenotypes are explained by and correlate with a strong reduction in *WUS* expression.

Additional findings support a role of chromatin regulation in control of *WUS* expression. For instance, variegated and ectopic expression of *WUS* is observed in loss-of-function mutations of FAS1 or FAS2 and BRU1, which are implicated in chromatin assembly and epigenetic silencing, respectively (Kaya et al. 2001; Takeda et al. 2004). The altered *WUS* expression is correlated with overproliferation and fasciation of the SAM. The data suggest that a restrictive epigenetic mark is present on the histone tails or on the DNA at the *WUS* locus in *WUS* nonexpressing cells of the SAM (but not in the organizing center), which is lost stochastically in these mutants, such that *WUS* transcription occurs in ectopic patches. However, the identity of this putative epigenetic mark and the reason for the requirement for FAS and/or BRU1 is currently not understood. In addition, the expression domain of *WUS* and *AG* is abnormally expanded in floral meristems in a mutant of AtGCN5, a histone acetyltransferase related to yeast GCN5 (Bertrand et al. 2003). It is unclear whether AtGCN5 directly restricts the expression of *WUS*, especially given that histone acetyltransferases usually act as transcriptional coactivators (Peterson and Workman 2000; Narlikar et al. 2002). Thus, spatially restricted expression of *WUS* in the SAM is controlled via chromatin regulation. In contrast, SYD is required for proper *WUS* transcript levels in its normal expression domain, the organizing center. *syd* mutants do not exhibit fasciation or ectopic *WUS* expression. A single focus of *WUS*:GUS-expressing cells was observed in the SAM of *clv1-4 syd-2* mutants. Both the domain of *WUS*-expressing cells and the *WUS*:GUS signal intensity per cell were reduced in the organizing center of *clv1-4 syd-2* compared with that of *clv1-4*. Furthermore, the organization of the cell layers in the SAM

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is not perturbed in *syd* mutants. These observations suggest that SYD regulates *WUS* expression differently from FAS1 or FAS2 and BRU1.

The finding that SYD is required for up-regulation of *WUS* transcription is consistent with the idea that SNF2-class ATPases are mainly involved in inducible or increased transcription (Narlikar et al. 2002). Two general models could explain the functional requirement for SYD in transcriptional up-regulation of *WUS* (Supplementary Fig. S1). In one scenario the nucleosomal DNA in the promoter region of *WUS* is not accessible to transcriptional activators or to the general transcriptional machinery in the absence of chromatin remodeling. In this case, the fact that SAM function is only impaired late in *syd*-null mutants is likely due to the activity of another, partly redundant, chromatin-remodeling activity. In this model, the second ATPase would provide the local change in nucleosomal structure in the *WUS* promoter in the absence of SYD, albeit less efficiently, thus resulting in lower *WUS* transcript levels. This model is supported by the fact that four SNF2 ATPases are present in *Arabidopsis* (Verbsky and Richards 2001). Moreover, the closest homolog to SYD, AtBRM, has recently been reported to play a role in SAM maintenance (Farrona et al. 2004). Alternatively, it is possible that the chromatin architecture of the *WUS* locus is partially “open” such that a low level of *WUS* expression is independent of ATP-dependent chromatin-remodeling activity. In this model elevated *WUS* transcription requires a structural change in the nucleosomal DNA driven by the SYD-containing chromatin-remodeling complex for enhanced binding of the same or of additional transcriptional activator(s). Both models are consistent with our observation that SYD is not required for *WUS* transcription under developmental conditions that do not require high levels of *WUS* message such as during the vegetative stage in the wild type. In addition, these models explain why *syd* causes SAM maintenance defects upon transition to reproductive development or under other conditions that may create or require increased levels of *WUS* transcript (in *clv1* or *wus/stm* mutants, respectively).

In summary, we show here that SYD is a direct and specific positive upstream regulator of *WUS* expression in the organizing center and that this activity is required for proper stem cell maintenance in the *Arabidopsis* shoot apical meristem.

Materials and methods

Plants

stm-1, *clv1-4*, *stm-2*, *wus-1*, and *syd-2* mutants have been described (Barton and Poethig 1993; Clark et al. 1993, 1996; Laux et al. 1996; Wagner and Meyerowitz 2002). Transgenic *Arabidopsis* seeds harboring *WUS*:GUS and *CLV3*:GUS were kindly provided by M. Lenhard and T. Laux (Institut für Biologie III, Universität Freiburg, Freiburg, Germany) (Groß-Hardt et al. 2002), and introgressed into the appropriate mutant background. Double-mutant plants were genotyped for *syd-2*, *wus-1*, and *stm-2* using dCAPS (Neff et al. 1998) and maintained as double heterozygotes. Heart-shaped cotyledons served as a visible marker for *syd-2* in young seedlings.

Arabidopsis seeds were sown on fertilized soil mix (Promix BX; Premier Horticulture), stratified in the dark at 4°C for 7 d and then grown at 22°C in long day (16 h light) at 120 μmol/m² sec of cool white light. Plants were photographed using an Olympus SZX12 dissecting microscope equipped with a Spot Insight camera (Diagnostic Inc.).

Reverse-transcriptase-mediated polymerase chain reaction (RT-PCR)

All above-ground plant tissues were used for RNA isolation. RNA isolation, reverse transcription, and PCR conditions were as described (William et al. 2004). PCR cycles varied from 26 to 36 depending on the message level. The PCR primers used are *WUS*, ACAAGCCATATCCCAGCTTCA and CCACCGTTGATGTGATCTTCA; *CLV1*, GCTTGCTAAGTTCTTAGTTGATGG and GCATCCGATGGCTGAGTTAT; *CLV3*, AATGGATCTAAAAGCTTTGTGCT and ACACCAAACGAAACAGATGTC; *POL*, GTACCACCGACCGTAGTGCT and GTGACGATCATGGAGCCTTT; *STM*, CATAACGAAATCGTTGCAGGA and ATCGACTTCTTCTCGGATGA; *AS1*, TGTGATGGAAGTTGCTCTTGA and ACCACTTCCCTAACCCTTT; *AS2*, TCCCATCTGTTTTGTTCCTA and TCTGAAGCTGACGAAGCTGA; *KNAT1*, TCTCCCTCAAACAACCGATAA and TGGGACTCTGACACAACATG; *KNAT2*, ACCACCGGAGACAATCAAAG and TCCGCTGCTATGTCATCATC; *CUC1*, CCACCTGGGTTTAGGTTTCA and AGAACCGCGAGAGAGAAGT; *CUC2*, TATCTTCCACCGGGTTTCAAG and CCGGTGCTAGCTAAAGTGGT; *CUC3*, CTCGAACACGACCATTCA and CGTTGAGCTGAGAGACGACA; *EIF4A*, AAACCTCAATGAAGTACTTGAGGGACA and TCTCAAAACCATAAGCATAAATACCC.

Cloning and sequencing of PCR product

An RT-PCR product harboring the *wus-1* mutation was subcloned into pGEM-T Easy vector (Promega), and sequenced using an automated sequencer. The *wus-1* mutation in the splicing acceptor site of the first intron resulted in the removal of the ~90-nt second exon by joining the donor site of the first intron to the acceptor site of the second intron (data not shown).

β-glucuronidase (GUS) assays and clearing of plant seedling

Arabidopsis seedlings were fixed overnight at room temperature in the solution of 90% ethanol and 10% acetic acid. After rehydration in 90%, 70%, and 50% ethanol solution for 20 min each, seedlings were cleared with Hoyer's medium (Liu and Meinke 1998). Cleared seedlings were photographed against a dark background using a Leica MZFLIII stereo microscope equipped with a CoolSnap camera (Photometrics Inc.). GUS staining and histological sections were performed essentially as described (Sieburth and Meyerowitz 1997). *Arabidopsis* seedlings harboring homozygous GUS transgenes were fixed in 90% ice-cold acetone, incubated with substrate for the durations indicated in the figure legends, and cleared for photography. For histological sections of GUS-stained plants, we used tertiary butanol instead of xylene. GUS signals were photographed using an Olympus BX51 compound microscope.

Scanning electron microscopy (SEM)

Tissue fixation and scanning electron microscopy were previously described (Chen et al. 2000). Samples were examined with a Jeol 6400 analytical scanning electron microscopy. Digital images were edited using Photoshop 6.0 (Adobe).

Western analysis

Nuclear extracts prepared from frozen plant tissues (Moebs et al. 1988) were dissolved in 1× Laemmli SDS sample buffer and run on 4%–12% gradient gel (Jule, Inc.). Membranes were probed with either anti-RNA polymerase II antibody generated against wheat germ Pol II (1:500; Covance) or SYD N-term-specific antiserum (1:10,000) (Y. Su and D. Wagner, unpubl.), followed by secondary probing with anti-mouse HRP (1:2,500) or anti-rabbit HRP (1:10,000), respectively. Secondary antibody-HRP conjugates and the detection kit (ECL plus) were from Amersham Biosciences.

ChIP

Buffers and procedures were essentially based on a manufacturer's protocol (Upstate). Plant tissue (~300 mg) was cross-linked in 1% formaldehyde solution under vacuum for 15 min. After incubation in 0.125 M glycine for 5 min, plant tissues were washed twice with 1× PBS, and subjected to preparation of nuclear extracts (Moebs et al. 1988). Nuclei were lysed in SDS Lysis Buffer (Upstate), diluted 10-fold with ChIP Dilution Buffer (Upstate), and sonicated to be a size range of 0.3–1.5 kb. Antibodies were prebound to protein A magnetic beads (DynaL Biotech) by overnight incubation at 4°C in 1× PBS with 0.5% BSA. Antiserum against LFY was described previously (William et al. 2004). Immunoprecipitation was performed in ChIP Dilution Buffer containing 2 µg/mL salmon sperm DNA for 16 h at 4°C on a rotating wheel. Beads were washed extensively twice each with Low Salt, High Salt and LiCl Immune Complex Wash Buffer and TE buffer (Upstate). Cross-linking of the eluates was reversed by incubation at 65°C overnight, and the precipitated DNA was purified with a PCR purification kit (Qiagen). One-twenty-fifth of the eluted DNA was used for subsequent PCR; the cycle number varied from 32 to 35. The primers used to assay for precipitation of genomic DNA were as follows; D (WUS), TCGATAGAAATGCCAAACGAC and GGATTCAA CACAACGTGCAA; P (WUS), TTTCAATCCTTCTCCACCA GA and GGGACCCAAAAAGAAGGTTT; G (WUS), GGCAAA CCAAATTTGCATCTA and TCAGTACCTGAGCTTGCAT GA; D (STM), GGTACAGGTGTCCCATGCTT and AACCAT TTGTATTTGCCACCA; P (STM), TCCGGTTTTCTCCATT TCAA and GAGGGAAGCAGCAGAAGAGA; G (STM), TTC CTTCAACGTGTGCGAGTG and TTGTTGTGTACCAAAG CAAATG; EIF4P, CGCATCCTATCGGATTGTCT and CTCA GATGATGTGCGGAGAA.

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