Noor Kornet

Role of chromatin factors in *Arabidopsis* root stem cell maintenance

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Rol van chromatine factoren in stamcelbehoud in de *Arabidopsis* wortel
(met een samenvatting in het Nederlands)

Proefschrift

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Promotor

Prof. dr. B.J.G. Scheres

Voor pap

Wetenschap is de titanische poging van het menselijk intellect
zich uit zijn kosmische isolement te verlossen door te begrijpen.

Willem Frederik Hermans
uit: Nooit meer slapen

Paranimfen

Anja van Dijken

Ilse Kornet

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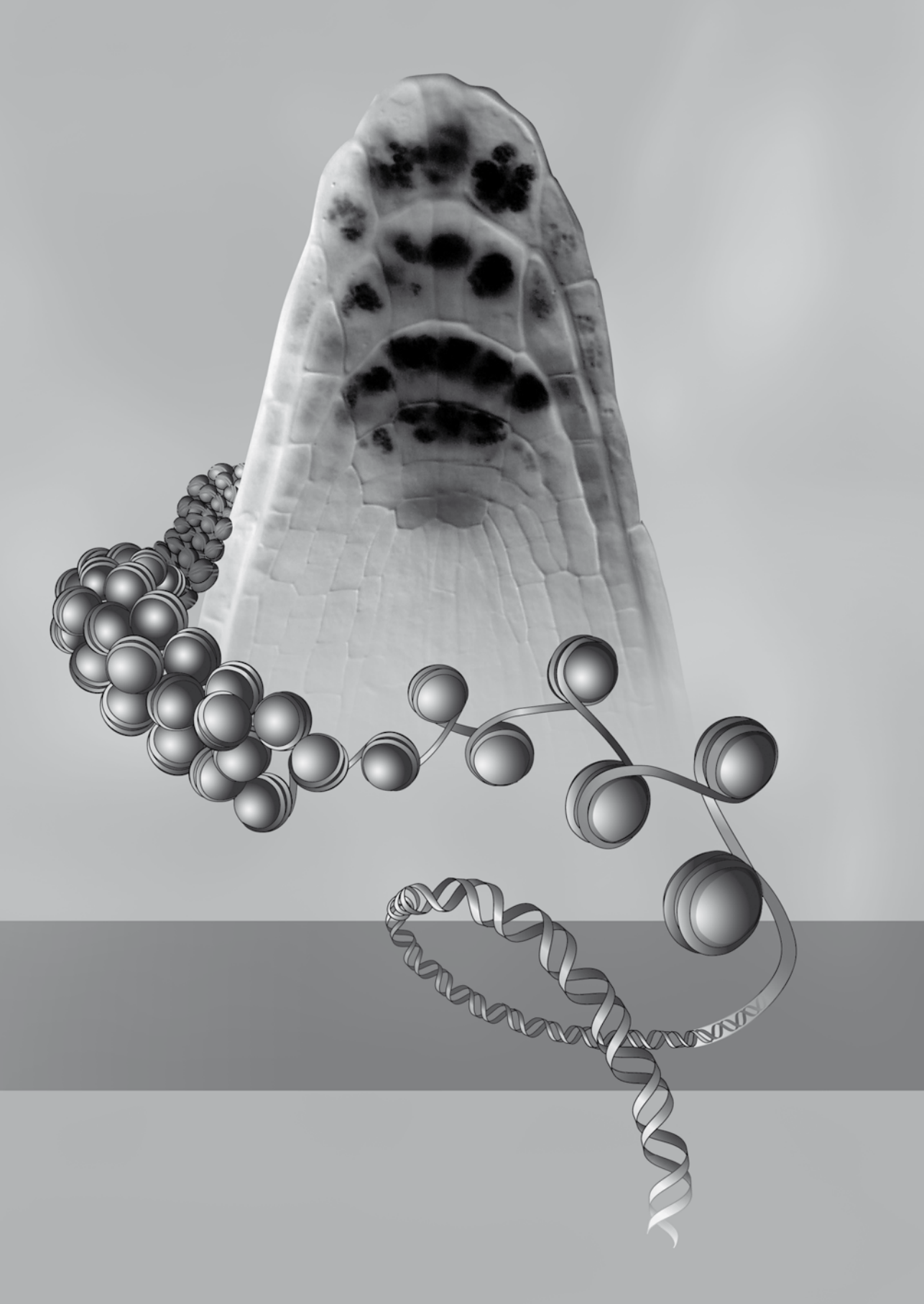
Contents



General introduction

CHAPTER 1

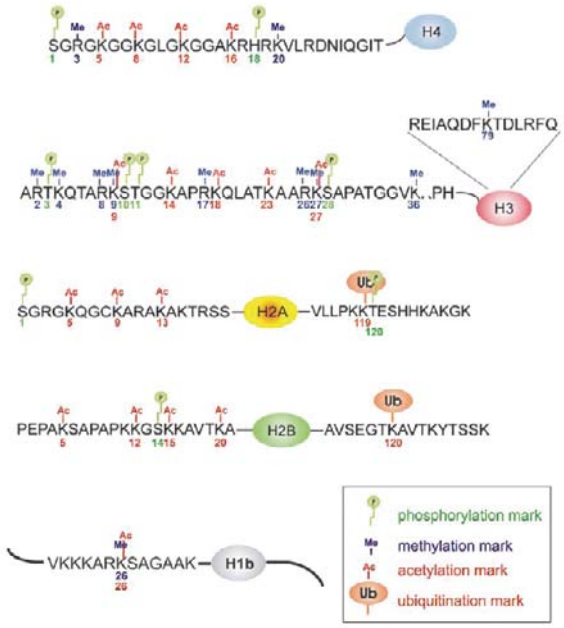
1 Introduction to chromatin

1.1 Chromatin structure

In the nucleus, DNA is compacted more than 10,000 times, while it still remains dynamic and accessible for transcription (reviewed by Luger and Hansen, 2005; Mellor, 2005). This is accomplished by histones, which form structures called nucleosomes. A nucleosome core particle consists out of 147 bp of DNA wrapped in 1.75 turns around a histone octamer core, containing 2 molecules of histones H2A, H2B, H3 and H4. Between the nucleosomes, 10-60 bp of 'linker' DNA is present, so that the nucleosomal array resembles a beads-on-a-string structure of approximately 10 nm in diameter. The array is compacted even further by linker histones, like H1, which are thought to stabilise the formation of a ~30 nm thick fiber (Bustin et al., 2005). The exact conformation of the 30 nm thick fiber is still under debate (Robinson and Rhodes, 2006; Tremethick, 2007) and the mechanisms of higher organisation of chromatin are less well understood.

The chromatin state can be influenced in four ways. First, methylation of the DNA can take place (Bernstein et al., 2007). Second, histone variants can be incorporated with different properties than the core histones (reviewed by Hake and Allis, 2006; Kamakaka and Biggins, 2005; Loyola and Almouzni, 2007). Third, sliding or replacement of nucleosomes can be facilitated by ATP-dependent chromatin remodellers (reviewed by Cairns, 2005; Kwon and Wagner, 2007). Fourth, both the nucleosomal core and the histone tails can be post-translationally modified: including lysine (K) acetylation, K and arginine (R) methylation, serine (S) and threonine (T) phosphorylation, K ubiquitination and sumoylation, ADP ribosylation, glycosylation, biotinylation, and carbonylation (Figure 1). These modifications can be placed or removed by enzymes, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), or histone methyltransferases (HMTs) and histone demethylases. Another level of complexity arises from the fact that lysines can be mono-, di-, or tri-methylated, and arginines can be mono- or di-methylated (the latter either in a symmetric or asymmetric manner) (reviewed by Bannister and Kouzarides, 2005; Kouzarides, 2007; Margueron et al., 2005; Peterson and Laniel, 2004; Shahbazian and Grunstein, 2007; Shi and Whetstine, 2007).

Figure 1 Post-translational modifications of histones



Histone residues are not only post-translationally modified in the histone tail, but also in the histone core. Sumoylation, ADP ribosylation, glycosylation, biotinylation, and carbonylation are not indicated, since relatively little is known about these modifications (adapted from Margueron et al., 2005).

1.2 Chromatin and transcription

Histone variants, remodellers, DNA methylation and histone modifications regulate genes in many ways. Certain histone variants, like H3.3, are found in transcriptionally active regions, while other H3 variants are found in silent chromatin (reviewed by Hake and Allis, 2006; Kamakaka and Biggins, 2005; Loyola and Almouzni, 2007). Chromatin remodellers can either activate or repress transcription by sliding, positioning or exchanging nucleosomes (reviewed by Cairns, 2005; Kwon and Wagner, 2007). Gene transcription is a tightly regulated process, which involves many

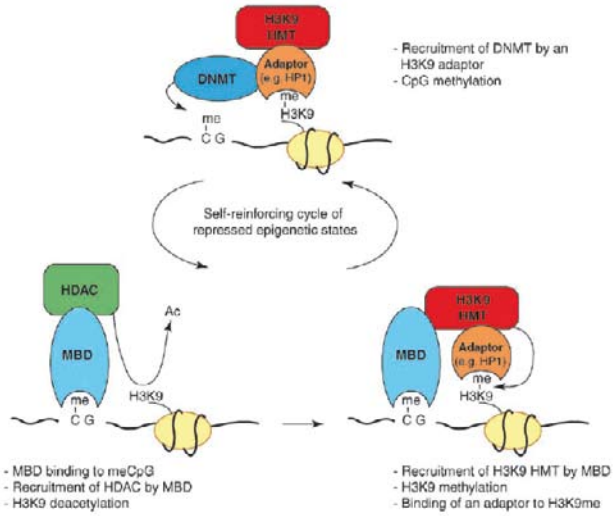
histone modifications (reviewed by Berger, 2007; Kouzarides, 2007; Li et al., 2007; Workman, 2006). Genome wide studies (mainly in yeast) show that some promoter elements contain nucleosome free regions. In general, at the transcriptional start of active genes, acetylation of histone H3 or H4 and trimethylation of lysine 4 of histone 3 (H3K4me3) are enriched. The HATs and HMTs responsible for these modifications are recruited by transcription factors and they facilitate transcription. An example is the yeast Gcn5 HAT containing complex SAGA, which is recruited by transcription factors and which is thought to 'loosen' chromatin at the promoter (Baker and Grant, 2007). H3K4me3 is also located at the 5'-end of the coding region and the HMT responsible for this modification is recruited by RNA polymerase II. In addition, the histone variant H3.3 is incorporated during elongation. At the 3'-end of the coding region, the elongating form of the RNA polymerase II recruits a HMT that mediates H3K36me3. This modification is recognised by a HDAC complex and histone deacetylation is thought to prevent transcription from cryptic promoter sequences present in the coding region. In general, inactive gene promoters posses DNA methylation, hypoacetylation of H3 and H4, H3K9me3 and/or H3K37me3, which are all modifications that repress transcription (reviewed by Berger, 2007; Kouzarides, 2007; Li et al., 2007; Workman, 2006).

1.3 The histone code

Post-translational modification of a histone tail not only influences other modifications on the same tail (in *cis*), but also affects the modifications on other tails (in *trans*) in the same or the neighbouring nucleosome (reviewed by Fischle et al., 2003b; Fuks, 2005; Kouzarides, 2007; Margueron et al., 2005; Nightingale et al., 2006). This has led to the 'histone code' hypothesis, stating that histone modifications, alone or in specific combinations, provide binding sites for chromatin factors that influence gene transcription (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2002).

How are chromatin factors recruited to their targets? This can be achieved by direct interactions with transcription factors in the case of transcription or by association to the basal transcriptional machinery. Another targeting mechanism involves small heterochromatic RNAs (shRNAs) which are produced by transcription of centromeric repeats and form double-stranded RNAs. These are processed by the RNAi machinery and the resulting siRNAs target chromatin factors to the centromere leading to hetero-

Figure 2 Self-reinforcing loop of DNA methylation, histone deacetylation and H3K9 methylation



Methylation of H3K9 by a histone methyltransferase (HMT) creates a binding site for an adaptor molecule (for example HP1), which recruits a DNA methyltransferase (DNMT). The DNMT performs DNA methylation, to which proteins with a methyl-CpG-binding domain (MBD) can bind. These MBD containing proteins recruit histone deacetylases (HDACs) (which prepare H3K9 for methylation) and HMTs (which methylate histones). Thus, the epigenetic code flows from histones to DNA and back, resulting in a epigenetic feedback loop (Fuks, 2005).

chromatin formation. Furthermore, non-coding RNAs can direct chromatin factors to specific locations on the DNA, for example during X inactivation (see section 2.1.2) (reviewed by Bernstein and Allis, 2005; Fischle et al., 2003b; Peterson and Laniel, 2004; Ruthenburg et al., 2007; Wassenegger, 2005).

The histone code is read by proteins with specific chromatin binding domains (reviewed by de la Cruz et al., 2005; Kouzarides, 2007; Ruthenburg et al., 2007). Examples are the chromodomain and bromodomain which bind methylated lysines and acetylated lysines, respectively. These domains are found in HATs, HMTs and remodellers, which in turn can modify chromatin and regulate gene transcription.

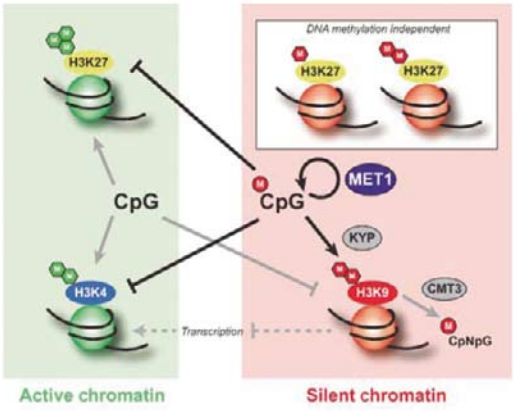
A well-known example of a self-reinforcing epigenetic loop, that maintains and perpetuates a heterochromatic state, is that of DNA methylation, followed by histone deacetylation and H3K9 methylation. H3K9 methylation in turn directs DNA methylation, resulting in a positive feedback loop (Figure 2) (reviewed by Fuks, 2005). This epigenetic loop might also be involved in spreading the silent state to neighbouring nucleosomes. A slightly different feedback loop between DNA methylation and histone methylation is found in *Arabidopsis* (Figure 3) (Lindroth et al., 2004; Mathieu et al., 2005). This suggests that variations of the histone code exist in different species.

1.4 Inheritance of the histone code

The histone code hypothesis implies that chromatin modifications are inherited through cell division to provide memory of a transcriptional state. But how are chromatin states maintained? DNA methylation is inherited semi-conservatively. After replication, hemi-methylated DNA is recognised by maintenance DNA methyl-transferases, the new DNA strand is methylated and thereby cellular memory is maintained. It is unclear whether histones are propagated after replication as tetramers and supplemented with newly synthesized histones or whether the parental H3/H4 tetramers are split into dimers and combined with newly synthesized H3/H4 dimers. In addition, it is unknown whether histone modifications are sufficient to impose the correct chromatin state. The feedback loop from DNA methylation to histone modifications is not essential, since the chromatin states are inherited correctly in species that lack DNA methylation (*Saccharomyces cerevisiae*) (reviewed by Groth et al., 2007; Martin and Zhang, 2007; Nightingale et al., 2006).

Histone variants have been proposed to contribute to the inheritance of the histone code. The histone barcode hypothesis stated that H3 variants contribute to cell lineage restriction and cellular memory through the incorporation of specific variants (Hake and Allis, 2006). This theory was confirmed (Ng and Gurdon, 2008) and contradicted (Loyola and Almouzni, 2007). Therefore the exact role of histone variants in epigenetic memory still remains to be elucidated. RNA was also suggested to play a role in chromatin inheritance, since it is known to target certain chromatin factors (Kouzarides, 2007). Alternatively, active chromatin may be permissive for transcription and when transcription is resumed after replication, the active histone modifications might be

Figure 3 Self-reinforcing loop of DNA methylation and H3K9 methylation in *Arabidopsis*



CpG methylation maintained by MET1 represses H3K27 trimethylation and H3K4 dimethylation, which are present in euchromatin. Conversely, methylated CpGs promote H3K9 dimethylation, which marks heterochromatin. The DNA methyltransferase CMT3 directly interacts with H3 when both H3K9 and H3K27 are methylated and subsequently performs CpNpG methylation. H3K9 and H3K27 are methylated by the histone methyltransferase KYP and by an unknown HMT, respectively (Mathieu et al., 2005).

reinstated on the new nucleosomes (Groth et al., 2007). Activating parental histone modifications, like acetylation and methylation, have been shown to 'survive' replication (Benson et al., 2006). Histone methylation and acetylation could be imposed on the new histones by multiple self-reinforced mechanisms. Recently, it was proposed that the chromatin state mainly determines nucleosome stability. After replication, transcription factors reinstate nucleosome mobility and thereby the active state, while silent chromatin is maintained during replication (Henikoff, 2008).

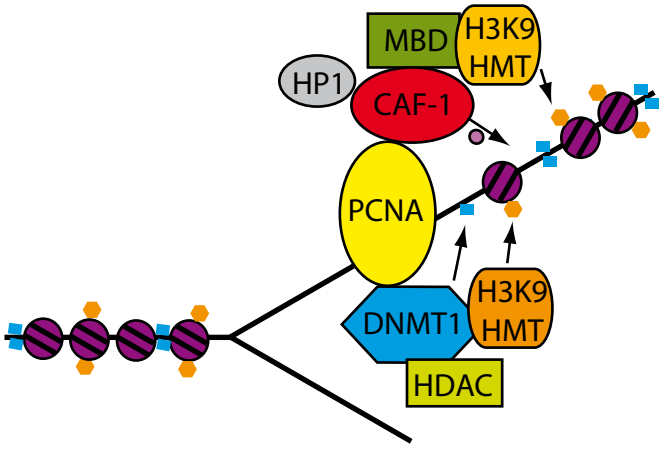
Silent chromatin is thought to be reinstated quickly after replication (reviewed by Groth et al., 2007; Wallace and Orr-Weaver, 2005). DNA methyltransferase DNMT1 is recruited to the replicated DNA by the replication factor PCNA and ensures the propagation of DNA methylation. DNMT1 recruits other chromatin factors, like HDACs and HMTs and in this way might help to replicate chromatin modifications (Figure 4) (Groth et al., 2007). PCNA recruits another chromatin factor, the CAF-1 complex (Shibahara and Stillman, 1999; Zhang et al., 2000), which is thought to remain

associated with heterochromatin for about 20 minutes after replication (Taddei et al., 1999). The chromatin assembly factor 1 (CAF-1) complex is involved in nucleosome assembly after replication by loading newly synthesized H3-H4 dimers onto the DNA. CAF-1 interacts with heterochromatin protein 1 (HP1) (Figure 4), which is known to be important for heterochromatin stabilization (Murzina et al., 1999), and may in this way facilitate chromatin maturation after replication (Quivy et al., 2004). However, there are redundant mechanisms, since HP1 can localize to heterochromatin independent of CAF-1 (Murzina et al., 1999). In addition to HP1, CAF-1 binds a DNA methyl binding (MBD) protein and a H3K9 HMT (Figure 4) (Reese et al., 2003; Sarraf and Stancheva, 2004). In turn, H3K9me3 is known to recruit HP1. Possibly, CAF-1 facilitates correct inheritance of chromatin modification through interaction with chromatin factors released ahead of the replication fork. Thus, both DNA methylation and histone methylation are immediately reinstated after replication. The self-reinforcing epigenetic loop between DNA methylation and H3K9 methylation (see section 1.3) and redundant mechanisms might ensure proper inheritance of silent chromatin (reviewed by Groth et al., 2007; Wallace and Orr-Weaver, 2005). In conclusion, although there are some clues, the exact mechanisms for inheritance of the chromatin state still need further investigation.

1.5 The histone code extended

Since the introduction of the histone code hypothesis, a lot of supplements and variants have been proposed. For example, the histone code hypothesis has been compared with models of signal transduction, which resulted in the signalling network model of chromatin (Schreiber and Bernstein, 2002). Since both systems need to integrate information, features of signal transduction models could also be relevant for chromatin regulation. The histone code hypothesis was also extended with the concepts of modification cassettes and binary switches (Fischle et al., 2003a). Modification cassettes are patches of modifiable sites, which might function as information units to mediate different signals, depending on their modification state. For example, in *Arabidopsis*, methylated H3K9 and H3K27 are simultaneously recognised by the DNA methyltransferase CMT3 (Lindroth et al., 2004). Alternatively, large complexes contain readers for different marks, providing combinatorial reading of multiple histone modifications (reviewed by Ruthenburg et al., 2007). The binary switch concept states that neighbouring modifications specify the read-out. For

Figure 4 Inheritance of the histone code



Replication factor PCNA recruits DNA methyltransferase DNMT1 to ensure proper inheritance of DNA methylation. DNMT1 in turn is known to be associated with HDACs and a H3K9 HMT. This ensures proper inheritance of histone methylation through an epigenetic feedback loop. In addition, PCNA recruits the CAF-1 complex involved in H3-H4 loading (small light purple sphere) to the newly replicated DNA. CAF-1 interacts with HP1 (which also can bind H3K9me3), a DNA methyl binding domain MBD protein and a H3K9 HMT (which ensures propagation of histone methylation). Blue squares depict DNA methylation, orange diamonds indicate histone methylation. For simplicity, only one strand is depicted.

example, HP1 binds trimethylated H3K9 (H3K9me3), unless H3S10 is phosphorylated (Fischle et al., 2005; Hirota et al., 2005). The nucleosome mobility model implies that the modifications present in the nucleosomal core might modulate histone-DNA interactions and in concert with nucleosome remodellers could change nucleosome mobility (Cosgrove et al., 2004). Histone modifications, like acetylation at H4K16, also disrupt higher order chromatin structures (Henikoff, 2005), independent of their interaction with chromatin factors (Shogren-Knaak et al., 2006). Recently, it was proposed in the chromatin scanning model that every site on the DNA is accessible due to the highly dynamic structure of chromatin (partial unwrapping of the nucleosome) and to protein mobility in the nucleus (van Holde and Zlatanova, 2006). This implies that transcription factors can access the DNA at all times and are able to 'read' and 'write' the histone code. The DNA sequence may in part define intrinsically the position of nucleosomes, which is thought to account for 30-50% of the *in vivo* nucleosome

positions in yeast and for 5% in eukaryotic genomes (reviewed by Morse, 2007; Rando and Ahmad, 2007).

In conclusion, all the different proposed models could be (partially) true and they may not be mutually exclusive. Importantly, chromatin modifications affect several processes. On the short term, chromatin factors influence ongoing processes like transcription. However, the chromatin state also sets a long-term context, for example in heterochromatin. Other chromatin modifications may be important for inheritance. A very important issue is to understand the long-term effects of chromatin modifications, which define different cell types (Turner, 2007). Therefore, we will have to examine the role of different chromatin modifications in all these processes on a gene-by-gene basis and we need to search in the future for the 'real' histone code defining the many different cell types known to date. Recent genome wide studies using novel techniques unravel the histone code further (Barski et al., 2007). Analysis of the chromatin state in different cell types with this technique will help unravel the 'real' histone code (Mikkelsen et al., 2007).

1.6 The *Arabidopsis* histone code

The *Arabidopsis* histone code is similar to that found in animals, although there are some differences (reviewed by Fransz et al., 2006; Gendrel and Colot, 2005; Hsieh and Fischer, 2005; Loidl, 2004; Pfluger and Wagner, 2007). First, in *Arabidopsis*, DNA methylation is not only present on CpG dinucleotides like in animals, but also on symmetric CpNpG sites and on non-symmetric CpNpN sites. In addition, unique maintenance DNA methyltransferases (CMTs), which contain a chromodomain, exist in plants to perform CpNpGp methylation. Second, a specific class of HDACs, called HD2, is present in plants, which is unrelated to the other HDAC classes. Third, *Arabidopsis* does not possess a Polycomb Repressive Complex 1 (PRC1) (see section 2.2.1). Finally, *Arabidopsis* heterochromatin contains H3K9me1/2 and H3K27me1/2 in contrast to H3K9me2/3 and H3K27me2/3 in animals. *Arabidopsis* euchromatin harbours H3K4me2/3, H3K9me3, H3K27me3 and H3K36me3 in contrast to H3K4me3 and H3K36me3 in animals. Together, this indicates that plants may 'speak a dialect' of the histone code. Recent genome wide studies are beginning to shed light on the complex role of histone modifications in *Arabidopsis* (Penterman et al., 2007; Turck et al., 2007; Zhang et al., 2007; Zhang et al., 2006; Zilberman et al., 2007).

2 Chromatin and development

Cell differentiation and development require extensive reprogramming of transcription patterns and this is mediated by chromatin factors. Some well-known examples of roles for chromatin in developmental processes are described below in both mammals and plants. In mammals, the role of chromatin in genomic imprinting, X inactivation and stem cell maintenance is reviewed. In plants, I discuss imprinting during endosperm development, flowering time control and stem cell maintenance.

2.1 Animal perspective

2.1.1 Mammalian genomic imprinting

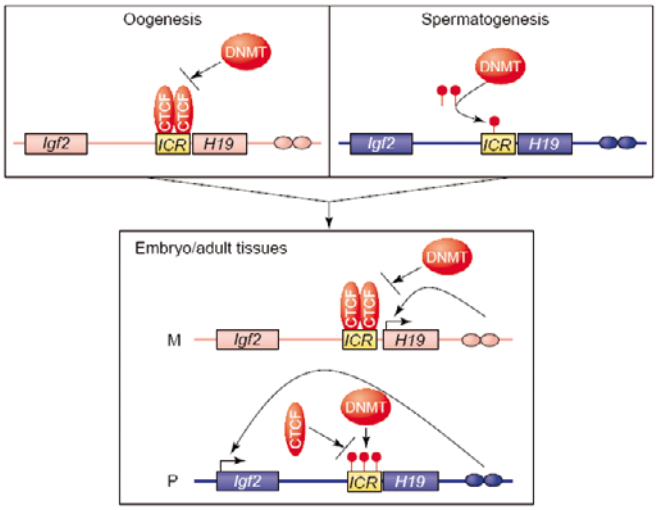
Imprinting is an epigenetic mechanism that regulates mono-allelic expression of a small subset of genes depending on their parent of origin. About 80 genes are now known to be imprinted in mammals and most are grouped in large chromosomal domains (reviewed by Delaval and Feil, 2004; Yang and Kuroda, 2007). Loss of imprinting is involved in a number of human diseases (Beckwith-Wiedemann, Prader-Willi and Angelmann syndrome) and is thought to play a role in cancer development. The maternal and paternal contributions to the developing embryo are different, since embryos containing only maternal or paternal chromosomes show developmental arrest and lethality. Imprinted genes associated with growth promotion, like *Igf2*, are expressed from the paternal allele, whereas those expressed from the maternal allele, like *Igf2r, Cdkn1c* and *Ascl2*, are growth suppressing. This suggests that imprinting has evolved from a conflict on maternal resources between the maternal and paternal genomes (described in the kinship theory or genetic conflict theory; Wilkins and Haig, 2003). Fathers are driven to extract the maximal resources for their offspring from the mother, while the mother benefits when her resources are divided equally among her offspring. A large amount of imprinted genes is involved in placental development, an important interface for transfer of maternal resources to the embryo. Imprinted genes are also involved in growth, embryonic development, and embryonic and adult brain development. When more resources are provided to the offspring, a resource conflict occurs between the parents, which may be resolved by an 'arms race' in genomic imprinting (reviewed by Constancia et al., 2004; da Rocha and Ferguson-Smith, 2004).

The imprinted domain is controlled by imprinting control regions (ICRs), which are rich in CpG islands (reviewed by Delaval and Feil, 2004). These ICRs are regulated by DNA methylation present on the CpG dinucleotide of either the maternal or paternal allele, which is thereby silenced. DNA methylation is known to regulate imprinting in two ways. First, DNA methylation of the paternal ICR prevents binding of a chromatin insulator protein (CTCF) and thereby influences the interaction of enhancer elements with either of two genes, for example at the *insulin-like growth factor-2 (Igf2)* and *H19* locus (Figure 5). Second, DNA methylation of the maternal ICRs prevents expression of non-coding RNAs, which cause allele-specific silencing. Two examples are the imprinted *Igf2r* and *Kcnq1* loci, which encode a IGF2 receptor and a cell-division regulator, respectively (Figure 6) (Delaval and Feil, 2004).

In the primordial germ cells (PGCs), the existing imprints are erased actively by unknown enzymatic activities (see also Figure 13, section 2.2.1). Later, during gametogenesis, novel imprints are established by the *de novo* DNA methyltransferase Dnmt3a. Methylation of the CpG islands is regulated differently during spermatogenesis and oogenesis to acquire sex-specific imprints. The binding of non-histone chromatin insulators (like CTCF) to the ICR could prevent methylation in one of the two germ lines, for example at the *Igf2* and *H19* ICR during oogenesis (Figure 5) (Fedoriw et al., 2004). Recently, it was found that maternally and paternally methylated ICRs contain different histone modifications during early spermatogenesis. These modifications might survive the large chromatin changes occurring during late spermatogenesis, possibly transmitting the imprint to the fertilized egg (Delaval et al., 2007). During pre-implantation development the male genome is actively demethylated. In addition, the whole genome is widespread passively demethylated and subsequently remethylated. It is unknown why ICRs are resistant to these large global methylation changes and how the imprints are maintained (Lewis et al., 2006). A protein containing a methyl-CpG-binding (MBD) domain binds and was suggested to protect the DNA methylation on the *Igf2* and *H19* ICR, since depletion of this MBD protein reduced DNA methylation and caused a reactivation of the *H19* gene (Reese et al., 2007).

Imprinting needs to be maintained during somatic development and this might be accomplished in several ways (reviewed by Delaval and Feil, 2004). First, continuous expression of the maintenance DNA methyltransferase Dnmt1 is essential (reviewed by Delaval and Feil, 2004). Second, chromatin insulator CTCF maintains imprinting by

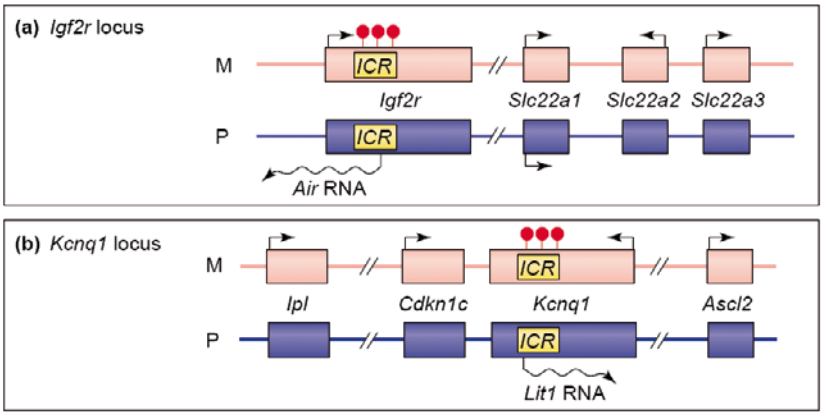
Figure 5 Establishment and maintenance of DNA methylation at the *Igf2-H19* locus



The paternal ICR acquires DNA methylation (red lollypops) during spermatogenesis. However, this is prevented during oogenesis by the binding of CTCF to the ICR. After fertilisation, CTCF binds the ICR of the maternal allele and prevents the interaction between the enhancer elements (ovals) and the *Igf2* gene. In contrast, the methylation of the paternal ICR prevents CTCF binding. Therefore, the *Igf2* gene interacts with the enhancers. M, maternal allele; P, paternal allele (Delaval and Feil, 2004).

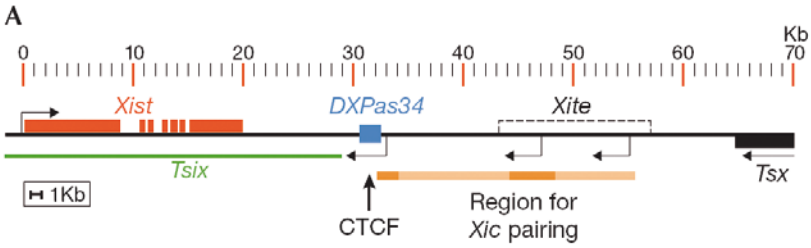
binding the unmethylated maternal allele and prevents aberrant DNA methylation possibly by forming a complex higher order structure (Fedoriw et al., 2004; Kurukuti et al., 2006; Lewis and Murrell, 2004; Rand et al., 2004). Third, binding of chromatin factors with methyl-CpG-binding domains (MBD) could protect the DNA methylation and recruit other chromatin factors (Reese et al., 2007). Finally, several redundant histone modifications are present at imprinted loci and thereby the silent or active state is maintained. For instance, not all imprinted alleles contain DNA methylation or are reactivated in *Dnmt1* mutant mice, indicating that different epigenetic mechanisms control imprinting (Caspary et al., 1998; Li et al., 1993). Repressive chromatin modifications can be detected on the silenced allele and the active allele contains an active chromatin state (Delaval et al., 2007; Lewis et al., 2004; Regha et al., 2007; Umlauf et al., 2004; Yamasaki et al., 2005). The Polycomb Repressive Complex 2 (PRC2), which

Figure 6 Non-coding RNAs and genomic imprinting



The non-coding RNAs *Air* (a) and *Lit1* (b) are expressed from the unmethylated paternal allele, but not from the methylated maternal allele (red lollypops). They are involved in silencing of the paternal allele of the imprinted *Igf2r* (a) and *Kcnq1* (b) locus, respectively. M, maternal allele; P, paternal allele (Delaval and Feil, 2004).

Figure 7 X inactivation centre



The X inactivation centre (Xic) is involved in counting and choice. The non-coding RNA *Xist* and the anti-sense transcript *Tsix* are shown. The position of the CTCF binding sites as well as the regulatory elements *Xite* and *DXPas34*, which are involved in pairing, is indicated (Ng et al., 2007).

is known to be involved in gene repression and possesses H3K27me3 activity, also plays a role at some imprinted loci (Mager et al., 2003).

In the embryo, imprinting is mostly maintained at genes that also contain DNA methylation. Some genes are only imprinted in the placenta, like *Ascl2*, encoding a transcription factor involved in placental development. Imprinting at these loci is independent of DNA methylation, but coincides with H3K9 and H3K27 methylation and the presence of the PRC2 complex. Chromatin repression in the extra-embryonic tissues is maintained by the ICR, similar to imprinting in the embryo (Lewis et al., 2004; Regha et al., 2007; Umlauf et al., 2004).

Taken together, several chromatin factors are involved in the maintenance of imprinting. DNA methylation is mainly used for imprinting in the embryo, while Polycomb repression by the PRC2 complex is mostly found on imprinted genes in extra-embryonic tissues like the placenta. The precise chromatin factors and their exact role during the initiation and maintenance of imprinting are just starting to be uncovered. In addition, the survival of the imprints during global developmental chromatin changes is not properly understood and this will need further investigation.

2.1.2 X inactivation

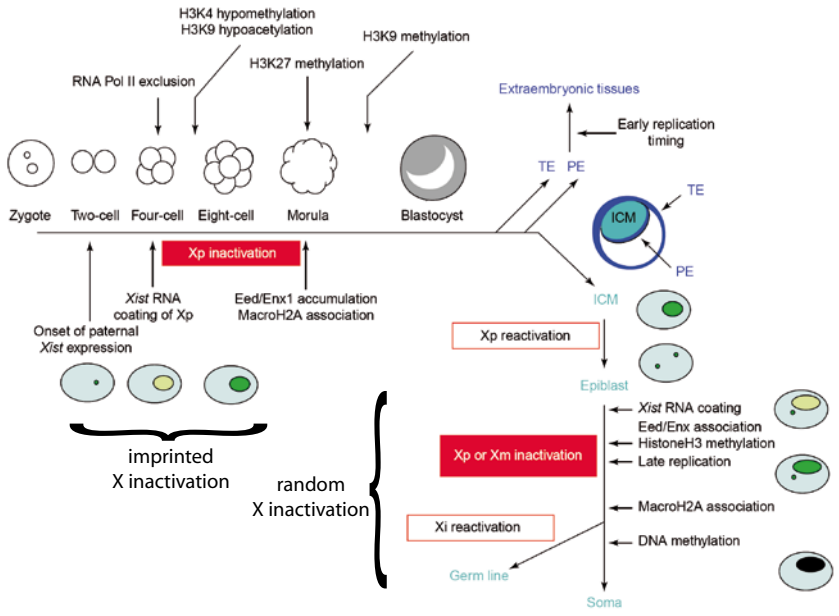
Dosage compensation mechanisms have evolved to equalise X-linked gene expression between males and females. In mammals, one X chromosome in the developing female embryo is inactivated to compensate the number of X chromosomes relative to the number of autosomes. X inactivation shows similarities to genomic imprinting and comparable epigenetic mechanisms may play a role. During genomic imprinting a small region is repressed, but in the case of X inactivation an entire X chromosome is silenced (for recent reviews: Clerc and Avner, 2006; Heard and Disteche, 2006; Ng et al., 2007; Wutz, 2007; Yang and Kuroda, 2007). The initiation of X inactivation is dependent on the X inactivation centre (Xic) (Figure 7) (reviewed by Latham, 2005). This locus is implicated in counting the number of X chromosomes in a cell and in the choice which X chromosome to inactivate. It encodes the non-coding RNA *Xist*, which is essential for the initiation of silencing. This resembles the silencing by the non-coding RNAs expressed from imprinted loci (see section 2.1.1). An antisense non-coding RNA, *Tsix*, represses *Xist* expression, acting as a binary switch.

Early in development, in the two-to-four-cell pre-implantation embryo, the paternal X chromosome (Xp) is preferentially inactivated: imprinted X inactivation (Figure 8). There are currently two theories that explain the preferential inactivation of the Xp and

they may not be mutually exclusive. First, the maternal copy of *Xist* may be inactivated in the early embryo by an unknown epigenetic mark that originates in the egg, keeping the maternal X chromosome (Xm) active (Reik and Ferguson-Smith, 2005). Second, the Xp is pre-inactivated, perhaps by epigenetic marks like DNA methylation and by the chromatin insulator CTCF, although this has to be examined further (Boumil et al., 2006). The Xp was also suggested to be pre-inactivated during spermatogenesis due to the repression of unpaired sex chromosomes (meiotic sex chromosome inactivation (MSCI)). However, when *Xist* is placed on autosomes, Xp inactivation still takes place (Okamoto et al., 2005). In addition, RNA signals from the Xp are present at the two-cell stage, indicating that the Xp is initially active and that silencing is spread from the Xic (Huynh and Lee, 2003; Okamoto et al., 2004). The need for the mother to silence the paternal X chromosome during early development and in the extra-embryonic tissues might lie in the kinship theory or genetic conflict theory (described in section 2.1.1), which states that a conflict on maternal resources exists between the maternal and paternal genomes (Wilkins and Haig, 2003).

The transcriptional silencing of one X chromosome is initiated by the non-coding RNA *Xist*, which is expressed exclusively from the future inactive X chromosome (Xi). The *Xist* RNA coats the Xi *in cis* and represses transcription by an unknown mechanism. Several chromatin changes occur on the Xi to provide an early maintenance of the silencing established by *Xist* (Figure 8) (reviewed by Heard, 2004; Heard, 2005; Heard and Disteche, 2006; Lucchesi et al., 2005; Nusinow and Panning, 2005). This includes loss of euchromatin-associated histone modifications, like H3K4me3 and H3K9Ac. Then, the Polycomb Repressive Complex 2 (PRC2) is recruited to the Xi, which contains HMT activity and is responsible for the trimethylation of H3K27 at the Xi (Silva et al., 2003). Along the Xi, H3K9 is dimethylated, possibly by the HMT G9a. To the H3K27me3 sites, a second Polycomb Repressive Complex (PRC1) is recruited, which mediates H2AK119 mono-ubiquitination (Plath et al., 2004). Recruitment of the complete PRC1 complex is not only dependent on the PRC2 complex, but *Xist* is also responsible for the recruitment of (part of) the PRC1 complex in a redundant fashion (Schoeftner et al., 2006). The PRC1 complex might silence target genes by interfering with the chromatin-remodelling machinery, blocking transcription initiation or recruiting additional silencing activities. Polycomb complexes are required for the initial phase of silencing, but not for the long-term maintenance of chromosomal memory. *Xist* expression during early differentiation efficiently establishes all chromatin

Figure 8 X inactivation during mouse development



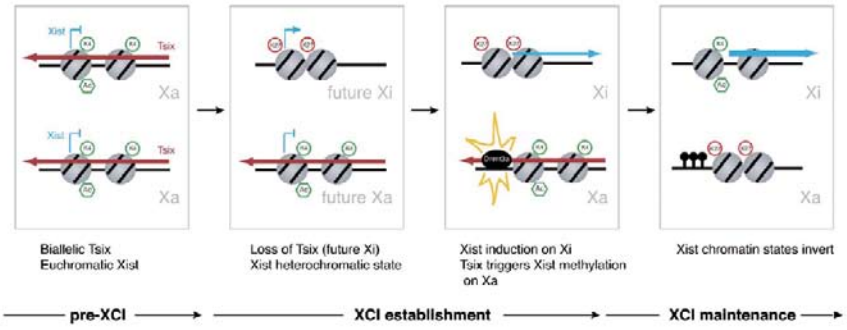
During early embryogenesis the Xp is preferentially silenced (imprinted X inactivation). The small green dot represents initial silencing of the Xi by *Xist*. This spreads through the entire Xi (yellow domain) and is maintained by recruitment of chromatin factors (green domain). Eed and Enx1 are the mouse homologues of PRC2 complex subunits Esc and E(z), respectively. Xp inactivation is reversed in the ICM, which will form the embryo. Subsequently, the X chromosomes are inactivated at random (random X inactivation) through a similar mechanism as the inactivation of the Xp. In the trophectoderm (TE) and primitive endoderm (PE), which are extraembryonic tissues, the Xp remains inactivated (adapted from Heard, 2004).

modifications, whereas *Xist* expression in differentiating cells is less efficient. In differentiated cells, a shift from reversible to irreversible silencing takes place. However, recruitment of the PRC2 and PRC1 complex by *Xist* is not sufficient for permanent silencing. A silencing defective form of *Xist* (lacking the A-repeats) is still able to recruit the PRC2 complex, induce H3K27 methylation and recruit the PRC1 complex, but this is not sufficient for maintenance of silencing (Plath et al., 2003; Plath et al., 2004; Silva et al., 2003). This suggests that there is redundancy or that unidentified mechanisms play a role in differentiated cells (Kohlmaier et al., 2004; Schoeftner et al., 2006; Wutz,

2007). Later in development, independent of *Xist*, the Xi is irreversibly silenced by the incorporation of the histone variant macroH2A and DNA methylation.

Later in development, during the blastocyst stage, the silenced Xp is reactivated in the inner cell mass (ICM), which will form the embryo (Figure 8). Subsequently, one X chromosome is inactivated at random, because *Xist* accumulates on this X chromosome (random X inactivation). *Tsix*, the antisense transcript of *Xist*, plays an important role in this choice. When the promoters of *Xist* or *Tsix* are mutated or when transcription through the locus is blocked, X inactivation is skewed. Recently, it was shown that the X chromosomes physically pair prior to the initiation of X inactivation and this is dependent on the presence of the Xic (reviewed by Turner, 2006). The distinction between the active and inactive X chromosome is established by histone modifications (Figure 9) (Sun et al., 2006). *Tsix* expression on both alleles maintains H3K4me2, which keeps *Xist* silent. By an unknown mechanism *Tsix* is downregulated on the future inactive X chromosome (Xi), which induces trimethylation of H3K27. Thereby *Xist* transcription is upregulated, in contrast to the general function of this heterochromatic mark to silence genes. *Xist* may belong to the rare class of genes that requires a heterochromatic environment to be expressed. *Tsix* RNA is found in a complex with DNA methyltransferase DNMT3 and DNA methylation can be found around the *Xist* promoter during the establishment phase. Since DNA methylation patterns are not significantly reduced in females with a *Tsix* deletion, this might serve as a secondary mechanism of silencing. During the maintenance phase of X inactivation, chromatin states are reversed according to their expression states (Figure 9) (Sun et al., 2006). Chromatin factors might be recruited by the RNA polymerase machinery, by the transcript or possibly by the RNAi machinery (Yang and Kuroda, 2007). Subsequently, *Xist* coats the Xi in *cis* and thereby initiates and maintains silencing by the recruitment of the PRC2 and PRC1 complexes as described above. Recently, exclusion of RNA polymerase and associated transcription factors from the *Xist* domain was found to be the earliest event after *Xist* accumulation. The formation of a silent nuclear compartment occurs prior to the observed changes in histone modification and occurs also in the silencing defective form of *Xist* (lacking the A-repeats). Subsequently, genes on the X chromosome are relocated into the *Xist* RNA compartment when they become silenced and this is dependent on the A-repeats of *Xist*. In addition, X-linked genes prone to escape silencing are found more frequently on the periphery of the compartment (Chaumeil et al., 2006).

Figure 9 Chromatin modifications at the X inactivation centre



Before inactivation *Tsix* is expressed on both alleles and is necessary to maintain H3K4me2. At the onset of inactivation *Tsix* is downregulated on the future inactive X chromosome and H3K27me3 accumulates. Thereby *Xist* transcription is upregulated. *Tsix* RNA recruits DNA methyltransferase DNMT3, which methylates DNA around the *Xist* promoter during the establishment of X inactivation. After X inactivation is completed, chromatin states are reversed. Xi, inactive X chromosome; Xa, active X chromosome (Sun et al., 2006).

In the extra-embryonic lineages, such as the placenta, the Xp remains inactive. The patterns of chromatin modifications differ slightly. For example, DNA methylation is absent in extra-embryonic lineages and the PRC2 complex is responsible for the cellular memory during differentiation (Heard, 2004; Kalantry et al., 2006; Lucchesi et al., 2005). This resembles the chromatin state in the placenta during genomic imprinting (described in section 2.1.1) and suggests a requirement for short-term maintenance of silencing.

Recently, two alternating and non-overlapping types of facultative heterochromatin were found to be present on the human X chromosome. One is characterised by *Xist*, macroH2A and H3K27me3, and the other by H3K9me3, HP1 and H4K20me3 (Chadwick and Willard, 2004). Another study confirmed the presence of redundant chromatin modifications (Barr et al., 2007). Thus, several redundant epigenetic silencing mechanisms are present to ensure efficient and secure silencing of the inactive X chromosome.

About 10-20% of human X-linked genes escape inactivation with great heterogeneity among females, while only a few mouse genes escape silencing (Chow et al., 2005). Accordingly, these genes lack some histone modifications characteristic for silent chromatin and instead contain active histone modifications. Escape is probably regulated at the level of chromatin domains. Boundary elements (containing binding sites for chromatin insulator CTCF) on either side of the escaping domain may mediate the formation of a chromatin loop positioning escape genes in a separate nuclear compartment (Filippova et al., 2005). In addition, one of the types of facultative heterochromatin on the X chromosome could be more prone to escape of silencing than the other. The need for escaping X inactivation remains unclear, but there are several speculations. Escaping genes might have homologues on the Y chromosome, removing the need to silence one copy in females. Alternatively, these genes could confer female specific functions.

X inactivation creates an inequality between autosomal genes and genes located on the X chromosome. Recently, it was shown that compensation is accomplished in somatic tissues of both males and females by increasing the expression from the active X chromosome through an unknown mechanism. This resembles the situation in *Drosophila*, where dosage compensation is achieved by upregulation of the single X chromosome in males, which is mediated by histone modifications (Nguyen and Disteche, 2006).

In conclusion, chromatin modifications are implicated in many aspects of X inactivation, such as the initiation and maintenance of the inactivation process and the escape from silencing. Chromatin modifications seem to serve as epigenetic memory of the transcriptional state of the X chromosome rather than to establish differences in expression during the initiation of X inactivation. Surprisingly, certain genes, like *Xist*, are upregulated in a heterochromatic context, emphasizing that there can always be exceptions to the (chromatin) rules. The parallels between genomic imprinting and X inactivation suggest that chromatin provides cellular memory and that similar epigenetic mechanisms may play a role in both processes.

2.1.3 Animal stem cells

Stem cells replenish the cells present in an organism throughout its lifetime (reviewed by Jones and Wagers, 2008; Li and Xie, 2005; Wong et al., 2005). They have two

unique characteristics: the capability to self-renew and to differentiate into (several) cell types. Many stem cells are localized in stem cell niches, where a limited number of 'organizer' cells maintain the stem cells by inhibiting differentiation and regulating their self-renewal. This is accomplished by signals, deriving from the organizer cells and perceived by the stem cells. In general, stem cells are physically anchored to their niche or are maintained on a population level.

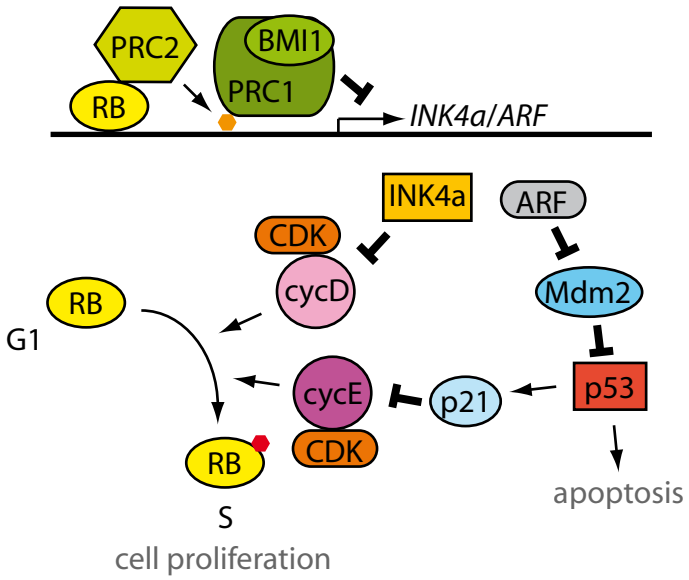
Mammalian embryonic stem (ES) cells, located in the inner cell mass of the embryo, are considered to be pluripotent, i.e. able to differentiate into all cell types. Adult stem cells, located in several tissues to provide maintenance and repair, are unipotent or multipotent, i.e. able to differentiate into one or a few cell types, respectively. Diverse pathways regulate stem cell maintenance depending on the stem cell niche, as different signals are found to be necessary to maintain the numerous stem cell types (reviewed by Jones and Wagers, 2008; Molofsky et al., 2004). Besides the influence of the stem cell niche microenvironment, stem cells are thought to possess unique intrinsic properties. The *Oct4*, *Nanog* and *Sox2* genes are essential intrinsically for the pluripotency and self-renewal of mammalian ES cells. *Oct4* encodes a POU domain transcription factor, *Nanog* a divergent homodomain protein and *Sox2* a HMG-box transcription factor. Analysis of the targets of Oct4, Nanog and Sox2 revealed that they regulate themselves, each other and key signalling pathways in stem cells and on the other hand repress key genes involved in differentiation. These data suggest that complex regulatory mechanisms are at work in ES cells, probably providing stability within the system by positive and negative feedback loops (Boyer et al., 2005; Johnson et al., 2006; Loh et al., 2006; Niwa, 2007a).

What makes stem cells unique? Except for the known stem cell factors Oct4, Nanog and Sox2, unique factors expressed only in stem cells are not found. This suggests that 'stemness' is not determined only by intrinsic factors, but also by the stem cell niche, as progenitor cells can revert to stem cells under certain conditions. However, not all cells are able to revert to a stem cell state, indicating that stem cells have unique features thought to be imposed by chromatin (reviewed by Buszczak and Spradling, 2006; Meshorer and Misteli, 2006; Mikkers and Frisen, 2005; Niwa, 2007a; Spivakov and Fisher, 2007). Accordingly, pluripotent ES cells contain hyperdynamic or 'breathing' chromatin, which is lost during differentiation. Furthermore, elevated levels of unbound histones in ES cells enhances differentiation, while ES cells expressing a

strongly bound version of H1 show differentiation arrest. This suggests that 'loose' chromatin is not only a unique trait of pluripotent cells, but that it also facilitates the structural chromatin changes occurring during the early stages of differentiation (Meshorer et al., 2006). Another study shows that major changes in chromatin organisation occur during early mouse embryogenesis. Heterochromatin is diffuse during the 2 cell stage and is progressively assembled into condensed heterochromatin until the blastocyst stage. Condensation of chromatin is abolished by deletion of the p150 subunit of the CAF-1 complex, which is involved in nucleosome assembly (Houlard et al., 2006). Heterochromatic modifications (H4K20me3 and H3K9me3) are reduced in ES cells by p150 depletion, then proliferation is affected and eventually cells die. Thus, the CAF-1 complex and extensive chromatin reorganisation are essential during the early steps of mouse embryogenesis and in ES cells (Houlard et al., 2006). Possibly, the CAF-1 complex is involved in the establishment of hyperdynamic or 'breathing' chromatin in ES cells.

The first chromatin factor shown to be an intrinsic stem cell factor is BMI1, a member of the Polycomb Repressive Complex 1 (PRC1). BMI1 is essential for the self-renewal of hematopoietic, neural, and cancer stem cells (reviewed by Valk-Lingbeek et al., 2004). BMI1 negatively regulates the *Ink4a/Arf* locus, encoding two cell cycle inhibitors, $p16^{ink4a}$ and $p19^{arf}$. Ink4a reduces CycD/CDK activity and thereby Retinoblastoma (RB) hypophosporylation, which induces cell cycle arrest (Figure 10). ARF inhibits degradation of p53 and activates apoptosis and cell cycle arrest (Lowe and Sherr, 2003; Sharpless, 2005). Therefore, BMI1 stimulates stem cell proliferation and prevents apoptosis. Deletion of the *Ink4a/Arf* locus only partially rescues stem cell self-renewal, indicating that BMI1 has additional unknown stem cell targets (Bruggeman et al., 2005; Molofsky et al., 2005; Valk-Lingbeek et al., 2004). Recently, it was shown that BMI1 binds the $p16^{ink4a}$ locus in a pRB family dependent manner in differentiated fibroblasts (Figure 10). The PRC1 complex, the PRC2 complex and H3K27me3 are enriched on the $p16^{ink4a}$ locus, which is abolished by reduction of pRB family proteins (Kotake et al., 2007). Binding of a PRC1 complex (containing BMI1) and of a PRC2 complex to the $p16^{ink4a}$ locus in differentiated fibroblasts was confirmed by another study and reduction of the PRC2 complex reduced binding of the PRC1 complex (Bracken et al., 2007). Taken together, this suggests that pRB family proteins recruit the PRC2 complex to the $p16^{ink4a}$ locus, which thereby obtains H3K27me3 and recruits the PRC1 complex (containing BMI1) to silence $p16^{ink4a}$ (Figure 10). In turn pRB family

Figure 10 Cell cycle regulation by Polycomb proteins and RB



At the *INK4a/ARF* locus, RB probably recruits the PRC2 complex, which mediates H3K27me3 (orange diamond). This in turn recruits the PRC1 complex containing BMI1 and represses transcription of the *Ink4a/Arf* locus. ARF inhibits degradation of p53 and thereby activates apoptosis and blocks cell cycle progression through p21 and cycE/CDK. INK4a inhibits cycD/CDK activity. The cycD/CDK complex promotes phosphorylation of RB (red diamond), which releases RB binding and thereby promotes the transition through the G1-S phase of the cell cycle.

proteins are regulated by *p16*^{ink4a}, forming a regulatory loop (reviewed by Lowe and Sherr, 2003; Sharpless, 2005). Possibly, elevated levels of BMI1 in stem cells ensure continuous proliferation by abolishing this feedback loop (Bracken et al., 2007; Kotake et al., 2007). RB was shown not to be required intrinsically in mouse hematopoietic stem cells (Walkley and Orkin, 2006), but instead was shown to be essential in the bone marrow microenvironment/niche (Walkley et al., 2007). Whether RB and Polycomb complexes act together on proliferation in ES cells similar to differentiated cells, needs further investigation. A Polycomb complex common to all stem cell types is not found, suggesting that distinct complexes regulate 'stemness'. BMI1 is mainly involved in the maintenance of adult stem cells, whereas probably other complexes are acting in ES cells (Valk-Lingbeek et al., 2004).

Recently, it was shown that a switch in subunits of the mammalian SWI/SNF chromatin remodeller is important for the transition from proliferating neural stem/progenitor cells to differentiated neurons. The neural stem/progenitor cell specific subunits are necessary and sufficient for proliferation (Lessard et al., 2007). Similarly, two chromatin remodelling factors from the SWI/SNF family were shown to be required for stem cell maintenance in the *Drosophila* ovary (Xi and Xie, 2005). Possibly, chromatin remodelling factors are required to maintain an 'open' chromatin configuration (hyper-dynamic chromatin). Alternatively, chromatin remodelling factors are also found associated with Polycomb complexes to maintain repression (possibly of differentiation genes). The exact role of chromatin remodellers and their mode of action in ES cells needs further investigation.

Recently, many genes involved in differentiation were shown to posses a special chromatin signature in mouse and human ES cells. They contain both active (H3K4me2/3 and H3K9 acetylation) and repressive (H3K27me3) modifications (reviewed by Buszczak and Spradling, 2006; Gan et al., 2007; Meshorer and Misteli, 2006). This implies that genes necessary for differentiation are kept silent in ES cells and are expressed at low levels, while being primed/poised for activation upon differentiation at the same time. These 'bivalent' domains are found exclusively in ES cells and are lost upon differentiation by resolving into either active (H3K4me3) or repressive (H3K27me3) chromatin. Interestingly, the domains overlap with highly conserved non-coding elements (HCNEs), which are found to be conserved among primates and rodents (Azuara et al., 2006; Bernstein et al., 2006). The PRC2 complex is responsible for H3K27me3 and repression in those domains, since differentiation specific genes are targets of PRC2 complexes and they are prematurely expressed in PRC2 mutant ES cells (Azuara et al., 2006; Boyer et al., 2006; Lee et al., 2006; Pasini et al., 2007). Interestingly, stem cell factors Oct4, Nanog and Sox2 are found specifically at a large subset of these differentiation genes. Therefore, stem cell pluripotency might be imposed by the intrinsic stem cell factors Oct4, Nanog and Sox2 through activation of a stem cell self-renewal program and a balance between activation and (a temporary) repression ('poised state') of differentiation factors by Polycomb complexes (Boyer et al., 2005; Lee et al., 2006). A genome wide map of chromatin modifications in pluripotent and differentiated cells confirmed the presence of bivalent domains in mouse ES cells and their resolution into either H3K4me3 or H3K27me3 in differentiated cell types (Mikkelsen et al., 2007). Recently, it was shown that the PRC1

complex is also present at bivalent genes and that H2AK119 mono-ubiquitination mediated by this complex keeps RNA polymerase II in a previously uncharacterised poised state by preventing transcriptional elongation (Stock et al., 2007). Differentiation into a specific lineage could be accomplished by selective activation of a subset of genes by lineage specific transcription factors, while other lineages of primed differentiation genes are permanently silenced. Resetting the repressed state could be achieved in several ways (reviewed by Schwartz and Pirrotta, 2007). First, by disrupting the balance between Polycomb and Polycomb antagonists, the Trithorax proteins. For example, Polycomb complexes are relocated to the nucleolus by tissue-specific TAFs (TATA binding protein (TBP)-associated factors) and subsequently Trithorax factors are recruited to the target promoter (Chen et al., 2005; Ringrose, 2006). Second, it was shown that transcription through the DNA binding site of Polycombs (Polycomb response element or PRE) influences Polycomb mediated silencing. Third, phosphorylation of Polycomb proteins causes them to dissociate from chromatin (reviewed by Schwartz and Pirrotta, 2007). Finally, it was found that the histone demethylase UTX specifically removes H3K27me2/3 from bulk chromatin and regulates many Hox genes. In addition, UTX associates with a complex containing H3K4 methylation activity (Agger et al., 2007; Lee et al., 2007). This suggests that Polycomb repression of Hox genes can be reversed by histone demethylases, which are assisted by H3K4 methylating complexes and in this way may resolve the bivalent domain into active chromatin. The recruitment of multiple histone modification activities could alter the epigenetic state and mediate appropriate differentiation. Although the theory of the poised state is appealing, the significance of bivalent domains needs to be further studied (discussed by Gan et al., 2007; Niwa, 2007a). Only a subset of key developmental genes contain bivalent domains and only half of these is occupied by Oct4, Nanog and/or Sox2 (and vice versa) (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2005; Boyer et al., 2006; Lee et al., 2006). In addition, binding of Polycomb factors does not always correlate with expression level (Bracken et al., 2006; Pasini et al., 2007). This illustrates that the deposition of bivalent domains is not yet fully understood and that the regulation of pluripotency may be more complex. Another point of interest is that PRC2 members are required to prevent stem cell differentiation and do not seem to affect proliferation (Azuara et al., 2006; Boyer et al., 2006; Niwa, 2007a; Pasini et al., 2007). Recently, it was proposed that bivalent domains facilitate postponement of cell fate decisions (Pietersen and van Lohuizen, 2008).

Recent data indicate that pluripotent transcription factors are essential and sufficient to induce the stem cell state. Overexpression of Oct4, Sox2, Nanog, c-Myc and Klf4 is sufficient to convert mouse somatic cells into induced pluripotent stem (iPS) cells, which show characteristics of stem cells (including chromatin features like bivalent domains and proper genomic imprinting) (Jaenisch and Young, 2008; Niwa, 2007a; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). Recently, it was shown that formation of iPS cells from adult human fibroblasts can be achieved with the same factors (Takahashi et al., 2007). This suggests that chromatin factors are not essential for pluripotency, but instead are involved in proper ES cell differentiation and possibly stabilization of the pluripotent or differentiation state. Therefore chromatin factors appear to be responsible for the 'execution' rather than the 'maintenance' of the pluripotent program, which is established by the pluripotent transcription factor network (reviewed by Niwa, 2007a). Oncogenes c-Myc and Klf4 are not essential, but increase the frequency of iPS cells (reviewed by Jaenisch and Young, 2008). The exact role of these factors in the reprogramming process is unclear. c-Myc (important for proliferation and stem cells) is required for the maintenance of active chromatin in progenitor cells. c-Myc regulates expression of the histone acetyltransferase *GCN5*, binds to the *GCN5* promoter and reduction of *GCN5* prevents the formation of active chromatin induced by Myc (Knoepfler et al., 2006). Therefore, c-Myc may provide access for transcription factors to target genes and aid in the reprogramming process (Jaenisch and Young, 2008).

Recently, it was shown that Oct4 binds the promoter and regulates the expression of histone demethylases *Jmjd1a* and *Jmjd2c* (Loh et al., 2007; reviewed by Niwa, 2007b). Jmjd1a and Jmjd2c bind and remove specifically H3K9 methylation from the *Tcl1* and *Nanog* promoter, respectively. Oct4 binding to the *Tcl1* promoter was abolished by *Jmjd1a* depletion (Loh et al., 2007). Thus, the pluripotent transcription factor Oct4 regulates the expression of chromatin factors *Jmjd1a* and *Jmjd2c*, which may play a role in maintaining open chromatin in stem cells. In turn, Jmjd1a mediates the access of Oct4 to the promoter of self-renewal regulator *Tcl1* (Loh et al., 2007; Niwa, 2007b).

In conclusion, a unique chromatin state is present in stem cells, which is imposed by transcription factors that program pluripotency. These transcription factors probably regulate chromatin factors to obtain a special chromatin state in ES cells. Polycomb proteins mediate the unique characteristics of stem cells: they regulate proliferation

and differentiation into several cell types. Distinctive (chromatin) factors might be important in different stem cell types (i.e. ES cells or adult stem cells) and this might reflect their (limited) potential to differentiate into several lineages.

2.2 Plant perspective

2.2.1 Imprinting and endosperm development in *Arabidopsis*

Upon fertilisation, one male gamete fuses with the plant egg cell to form the embryo. The other male gamete fertilises the homodiploid central cell leading to the development of triploid endosperm. The endosperm sustains embryonic development and provides reserve storage (reviewed by Berger et al., 2006; Huh et al., 2008; Kohler and Makarevich, 2006). Endosperm development must be exactly coordinated with embryonic development and is therefore tightly controlled.

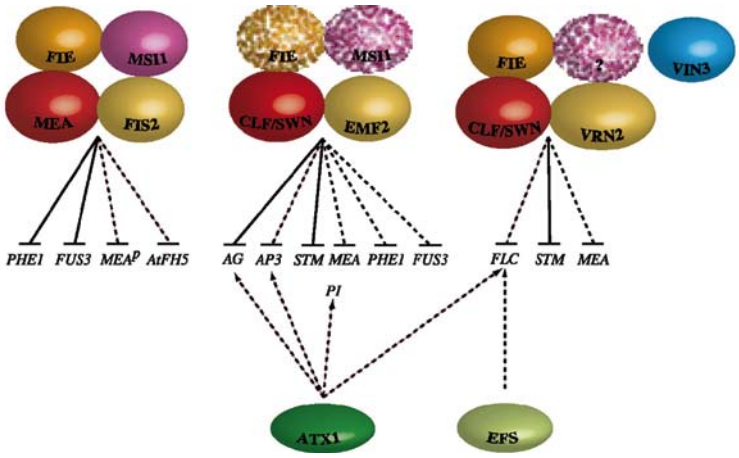
The *FERTILISATION INDEPENDANT SEED (FIS)* genes have an important role in repressing endosperm development. Endosperm proliferation and early development takes place without fertilisation in *fis* mutants. When fertilisation takes place in *fis* mutants, embryonic development is arrested and the seed aborts. Mutation of *FIS* genes cause a change in the timing of endosperm development (Ingouff et al., 2005). The *FIS* genes include *MEDEA (MEA)* / *FIS1, FERTILISATION INDEPENDENT ENDOSPERM (FIE)* / *FIS3* and *FIS2*, encoding homologues of the mammalian Polycomb group genes E(z), ESC and Su(z)12, respectively. In animals, Polycomb group genes E(z), ESC and Su(Z)12 form a complex, called Polycomb Repressive Complex 2 (PRC2), which represses transcription of target genes (reviewed by Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007). A similar complex likely exists in plants, consisting of MEA, FIE and FIS2, together with MSI1, a histone chaperone found in many chromatin complexes (also in animals) (Hennig et al., 2005; Kohler et al., 2003a; Spillane et al., 2000; Yadegari et al., 2000). In general, *Arabidopsis* PRC2 complexes have been duplicated and are involved in different aspects of plant development (Figure 11) (Calonje and Sung, 2006; Chanvivattana et al., 2004; Pien and Grossniklaus, 2007; Schubert et al., 2005). For example, the FIE, CLF/SWN, MSI1, EMF2 complex is involved in the repression of floral homeotic genes (*AG* and *STM*) and directly binds their promoter during embryonic and vegetative development (Schubert et al., 2006). Another PRC2 complex containing VRN2, a SU(Z)12 homologue, controls the floral repressor *FLC* through the vernalization

pathway (see section 2.2.2). Like their animal counterparts, the plant E(z) homologues SWN and CLF are implicated in H3K27me2/3 in euchromatin (Lindroth et al., 2004; Makarevich et al., 2006; Schonrock et al., 2006a). Recently, the SET domain of CLF was shown to be important for dispersed H3K27me3 and localised H3K27me2 on target promoters (Schubert et al., 2006). Furthermore, both H3K9me2 and H3K27me2 are reduced in *vrn2* mutants (see also section 2.2.2) (Bastow et al., 2004; Sung and Amasino, 2004). MEA is necessary for deposition of H3K27me2/3 (see also below) (Gehring et al., 2006; Jullien et al., 2006a; Makarevich et al., 2006).

Although the homologous PRC2 subunits are involved in different aspects of development, there is also redundancy. The *vrn2 emf2* and *clf swn* double mutants have a much more severe phenotype than the single mutants (Chanvivattana et al., 2004; Lindroth et al., 2004; Makarevich et al., 2006; Schubert et al., 2006). There is conflicting evidence on whether SWN and MEA act partially redundant during endosperm development (Spillane et al., 2007; Wang et al., 2006). The *swn clf* double mutant produces normal seeds (Chanvivattana et al., 2004). Therefore, only MEA is active during seed development. In animals, the PRC1 complex is recruited to the H3K27me3 sites deposited by the PRC2 complex and provides stable silencing. No plant homologues of the PRC1 complex have been found. Therefore, Polycomb complex mediated silencing might be less stable in plants or proteins different from those found in the animal PRC1 complex might be recruited to provide stable repression (see also section 2.2.2). In animals, Polycomb action is counteracted by Trithorax genes, encoding chromatin remodellers or histone methyltransferases (reviewed by Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007). Evidence for functional *Arabidopsis* Trithorax homologues is now available. ATX1 possesses H3K4me3 activity *in vitro* and expression of floral homeotic genes (*AG, PI* and *AP3*) is reduced in *atx1* mutants (Alvarez-Venegas et al., 2003). It has been suggested that ATX1 also maintains *FLC* expression via H3K4me3 of *FLC* (Pien and Grossniklaus, 2007). Another putative *Arabidopsis* Trithorax protein is EFS/SDG8, which affects either H3K4me2 or H3K36me2 at *FLC* chromatin (see also section 2.2.2) (Kim et al., 2005; Zhao et al., 2005). However, a role for Trithorax proteins in seed development is not yet uncovered.

The effect of *fis* mutations is only apparent when inherited through the female gamete, irrespective of the paternal allele. All three *FIS* genes are expressed in the central cell

Figure 11 Target genes of *Arabidopsis* PRC2 complexes



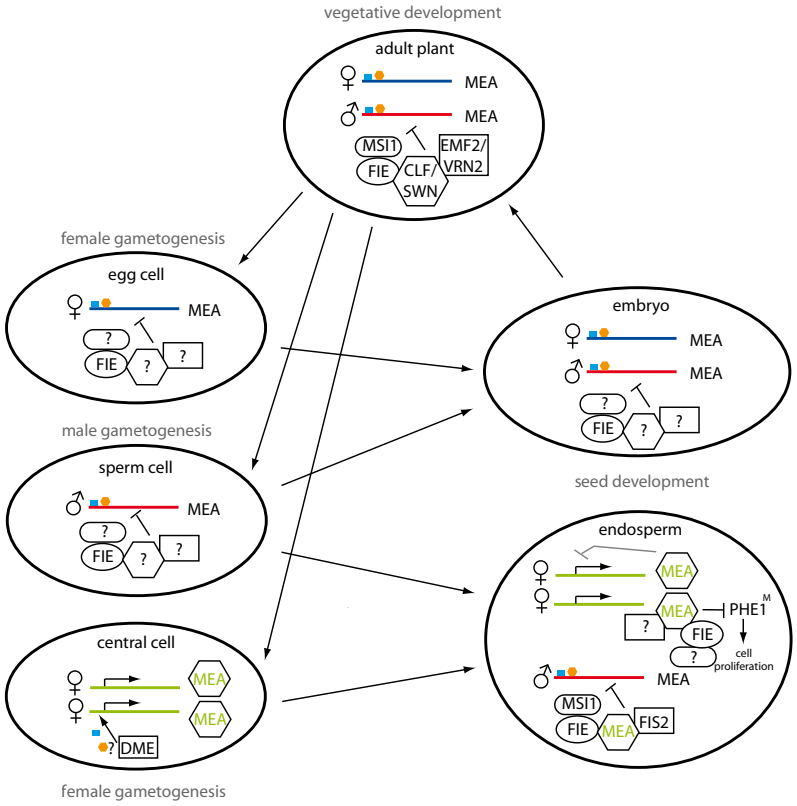
In *Arabidopsis*, there are at least three PRC2 complexes with different expression patterns and divergent functions. The MEA complex is present during gametogenesis and seed development, the EMF2 complex is present during flower development, the VRN2 complex is present during vegetative development. The colours indicate homologous *Drosophila* subunits: red, E(Z) homologue; yellow, SU(Z)12 homologue; orange, ESC homologue; pink, p55 homologue (MSI proteins). Light and dark green, Trithorax homologue. Fully coloured proteins have been shown to interact in the complex, while patchy coloured proteins are depicted when supported by genetic data. Solid lines indicate direct binding, dashed lines are based on genetic evidence. The paternal allele of MEA (MEA^p) is repressed by the MEA complex, while the maternal MEA allele is repressed by itself outside the PRC2 complex context (see text section 2.2.1). (Pien and Grossniklaus, 2007)

(endosperm precursor cell) before fertilisation. *MEA* is imprinted in the endosperm: it is exclusively transcribed from the maternal allele in the endosperm and the paternal allele is silenced (Figure 12) (reviewed by Autran et al., 2005; Berger, 2004; Schubert and Goodrich, 2003; Scott and Spielman, 2004). Gene expression in both embryo and endosperm is largely contributed by the maternal genome until three to four days after fertilisation. In addition, the active maternal *MEA* allele provides maternal control over seed development through its repressive properties. This supports the kinship theory or genetic conflict theory (see section 2.1.1), which states that a conflict on maternal resources exists between the maternal and paternal genomes (Wilkins and Haig, 2003). Imprinted genes involved in a parental conflict are thought to undergo positive

selection. Accordingly, MEA (but not the closest relative SWN) undergoes positive darwinian selection in the out-crossing species *Arabidopsis lyrata*. This is not the case in the self-fertilizing species *Arabidopsis thaliana*, which is thought to have weaker selective pressures (Spillane et al., 2007).

The DNA methyltransferase responsible for the maintenance of DNA methylation at the silenced *MEA* promoter is MET1 (Xiao et al., 2003). DEMETER (DME), a DNA glycosylase domain protein, is required to activate the transcription of *MEA* in the central cell, which will contribute to the endosperm. Only the maternal allele is exposed to DME activity in the central cell (Choi et al., 2002). It was shown that DME possesses DNA glycosylase activity and excises the DNA methylation (at CpG, CpNpG and CpNpN sites) present on the maternal *MEA* allele in the central cell (Gehring et al., 2006). Imprinting at *MEA* is therefore controlled by antagonism between MET1 and DME (Figure 12). In mammals the default state is active and imprinting is an active process, while in plants the default state is silent and imprints are actively erased (Figure 13) (reviewed by Feil and Berger, 2007). Surprisingly, the paternal *MEA* allele in the endosperm is not activated by hypomethylation in *met1* mutants. Instead, the maternal MEA-FIE complex is required for the maintenance of paternal *MEA* repression in the endosperm through H3K27me2 and independent of DNA methylation (Figure 12) (Arnaud and Feil, 2006; Gehring et al., 2006; Jullien et al., 2006a). In addition, the maternal *MEA* allele represses directly its own expression around fertilization, since maternal *MEA* transcripts accumulate higher in *mea* mutants than in wildtype and MEA is present at its own promoter. Maternal *MEA* transcripts are not elevated in *fis2, fie* and *msi1* mutants, therefore maternal *MEA* silencing is independent of the MEA-FIE complex (Figure 12). A lot of auto- and cross-regulation is found among PRC2 complex members around the time of fertilization, suggesting that the mechanism is even more complex (Baroux et al., 2006). During vegetative development, *MEA* is also repressed on both alleles by other PRC2 complexes (Jullien et al., 2006a). Thus, Polycomb repression maintains silencing of *MEA* throughout development, independent of DNA methylation (Figure 12). Although *MEA* is not reactivated in *met1* mutants, DME is necessary to activate *MEA* in the central cell (Gehring et al., 2006). Possibly, DME removes both DNA and histone methylation or loss of DNA methylation may be a prerequisite to remove histone methylation. Alternatively, DME may release a gene imprinted by DNA methylation, which controls histone methylation on *MEA*. It remains unknown how the MEA complex recognizes specifically the paternal allele to repress

Figure 12 Imprinting of *MEA* during development



Repression of both the maternal and paternal *MEA* allele is maintained by different PRC2 complexes throughout development. This is the CLF/SWN PRC2 complex during vegetative development and the maternal MEA PRC2 complex during seed development. The maternal allele of *MEA* is reactivated in the central cell by DME through removal of DNA methylation (blue squares) and possibly H3K27 methylation (orange circles). The MEA/FIE complex containing maternal MEA (green) represses the maternal allele of *PHE1* in endosperm and the paternal allele of *MEA*. In addition, maternal MEA moderately reduces its own expression independent of the rest of the complex. How the MEA complex recognizes specifically the paternal allele of *MEA* and the maternal allele of *PHE1* is unknown, but this is probably established in the central cell by DME.

its transcription in the endosperm, while the maternal *MEA* allele remains active. DNA methylation is only relevant in the central cell and also DME activity there is essential

for activation of *MEA* (Gehring et al., 2006). This suggests that DME activity in the central cell controls expression of the maternal allele and thereby silencing of the paternal allele after fertilization. However, the exact molecular mechanisms is unclear. Together, this resembles the situation in the mammalian placenta, where imprinting of both the genome and the inactive X chromosome is maintained by the PRC2 complex and H3K27 methylation independent of DNA methylation (see section 2.1.1 and 2.1.2).

DNA methylation does play an important role in endosperm imprinting at the *FIS2* gene. *FIS2* expression is only detected from the maternal allele and is dependent on DNA methylation by MET1. In *dme* mutants, *FIS2* expression is partially reduced, suggesting that DME is in part required for the activation of *FIS2* in the central cell. Probably redundant factors are acting at the *FIS2* promoter (Jullien et al., 2006b).

As mentioned before, the effect of the *fis* mutations is only found when inherited through the female gamete, independent of the paternal allele. This can be explained in the case of *MEA* and *FIS2*, because they are imprinted and expressed only maternally. Although *FIE* and *MSI1* are not imprinted, they are specifically expressed in the female gametophyte and in the very early endosperm only the maternal alleles are expressed (Leroy et al., 2007; Yadegari et al., 2000). Expression of the paternal allele of *MSI1* very early after fertilization is unable to rescue the *msi1* female gametophytic defect, but expression of *MSI1* in the female gametophyte rescues all aspects of the *msi1* phenotype. This suggests that a functional FIS complex is required in the female gametophyte (Leroy et al., 2007).

A direct target of the MEA/FIE complex is *PHERES1 (PHE1)*, encoding a MADS-box transcription factor. Normally, *PHE1* is expressed transiently after fertilisation in both the embryo and the endosperm, but it remains upregulated in the *mea* and *fie* mutants (Kohler et al., 2003b). Accordingly, the MEA-FIE complex binds directly to the *PHE1* promoter, repressing its expression (Figure 12). Reduction of *PHE1* expression suppresses *mea* seed abortion, suggesting that PHE1 is largely contributing to the overproliferation of endosperm (Kohler et al., 2003b). *PHE1* is paternally expressed after fertilisation, but is maternally repressed by MEA (Kohler et al., 2005). Probably, the MEA complex targets the maternal *PHE1* allele in the central cell or shortly after fertilization. The MEA SET domain and a PRC2 complex containing CLF or SWN are responsible for the H3K27me3 at the *PHE1* promoter in endosperm and leaves,

respectively (Makarevich et al., 2006). This suggests that different Polycomb complexes repress common targets during the different stages of plant development. Repression of maternal *PHE1* by maternal MEA supports the kinship theory or genetic conflict theory (see section 2.1.1) that describes that growth-promoting genes are expressed from the paternal genome, while they are silenced by the maternal genome (Wilkins and Haig, 2003).

Surprisingly, mutation of the SWI/SNF chromatin remodeller *DDM1*, rescues *mea* mutants, but not *fie* mutants. This, together with the fact that *MEA* is imprinted and *FIE* not, suggests that the separate components of the PRC2 complex have different roles during endosperm development (Yadegari et al., 2000). Targets of MEA might be affected by the mutation of *DDM1* and cause a rescue of seed abortion. Accordingly, *PHE1* is lower expressed in *ddm1 mea* double mutants than in *mea* mutants alone. DDM1 is implicated indirectly in modulating DNA methylation and histone methylation, although the exact mechanism is unknown (Kohler et al., 2003b). Imprinting during seed development can also be bypassed by another mechanism. Pollen with mutated cell cycle regulator *CDKA;1* only fertilize the egg cell, resulting in diploid maternal endosperm (Nowack et al., 2006). When *mea*, *fis2* or *fie* plants were crossed with *cdka;1* pollen, embryonic development was rescued and this resulted in plants producing viable offspring. *PHE1* expression was reduced in these plants compared to *mea*, indicating that gene dosage is important for seed viability. This suggests that the *FIS* genes are necessary to counteract the contribution of the paternal genome, which is consistent with the kinship theory or genetic conflict theory (Nowack et al., 2007).

Interestingly, imprinting in plants appears to be accomplished through a fundamentally different mechanism than mammalian imprinting (Figure 13) (reviewed by Feil and Berger, 2007). In animals, the default state is active and imprinting provides inactivation of a certain allele during gametogenesis, which is reset in the primordial germ cells. On the contrary, in plants, the default state is silent in the embryo and the silent allele needs to be activated only in the endosperm, which is not propagated. In plants imprinting is only found in the endosperm, while mammalian imprinting occurs both in the placenta and the embryo. The role of DNA methylation and histone methylation in imprinting is evident in both plants and mammals. In conclusion, *Arabidopsis* PRC2 complexes are involved in imprinting, endosperm development and several other

aspects of plant development. Similarities can be found between plant and animal Polycomb complexes. Nevertheless, some aspects are different, such as the presence of multiple PRC2 complexes and the absence of the PRC1 complex in plants.

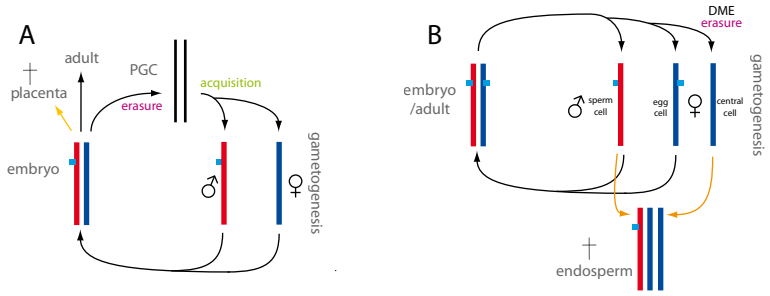
2.2.2 Flowering-time control in *Arabidopsis*

Flowering, the transition from vegetative to reproductive development, is a major developmental switch in the plant life cycle (reviewed by Boss et al., 2004; He and Amasino, 2005; Henderson and Dean, 2004; Schmitz and Amasino, 2007). Therefore, expression of genes involved in flowering must be tightly regulated. Four major pathways control the timing of flowering in *Arabidopsis* (Figure 14). The photoperiod pathway accelerates flowering in response to an increase in day length. Prolonged exposure to cold promotes flowering through the vernalization pathway. The autonomous pathway constitutively represses flowering and gibberellins induce flowering. These pathways regulate a set of target genes, the flowering-time integrators, such as *SOC1* and *FT*. These integrators activate floral-meristem identity genes, like *LEAFY* and *AP1*, promoting floral development. The vernalization pathway and the autonomous pathway converge on *FLC*, which encodes a MADS-box transcriptional repressor (Figure 14). In addition, FRI, which is found in late flowering *Arabidopsis* accessions, elevates *FLC* expression to inhibit flowering. Recently, several proteins in these flowering time regulating pathways were shown to mediate chromatin modifications at the *FLC* gene.

For example, the autonomous pathway contains *FLD* and *FVE*/*MSI4*, which encode components of a putative histone deacetylase (HDAC) complex. FLD and FVE are necessary for the deacetylation of *FLC* chromatin around the promoter, and thereby repress *FLC* transcription constitutively. The HDAC responsible for the actual deacetylation remains unknown (Ausin et al., 2004; He et al., 2003). In addition, *FLD* encodes a putative H3K4 demethylase, which is partially redundant with its homologues *LDL1* and *LDL2* (Jiang et al., 2007). Another putative histone demethylase, REF6, also represses *FLC* (Noh et al., 2004). Further investigation of chromatin modifications and the enzymatic activities of these chromatin factors are needed to assess their exact roles in constitutive *FLC* repression.

Furthermore, FRI, encoding a novel protein, induces H3K4 trimethylation of *FLC* chromatin around the promoter, which is a hallmark of active chromatin. This abolishes

Figure 13 Mechanism of imprinting in mammals and plants

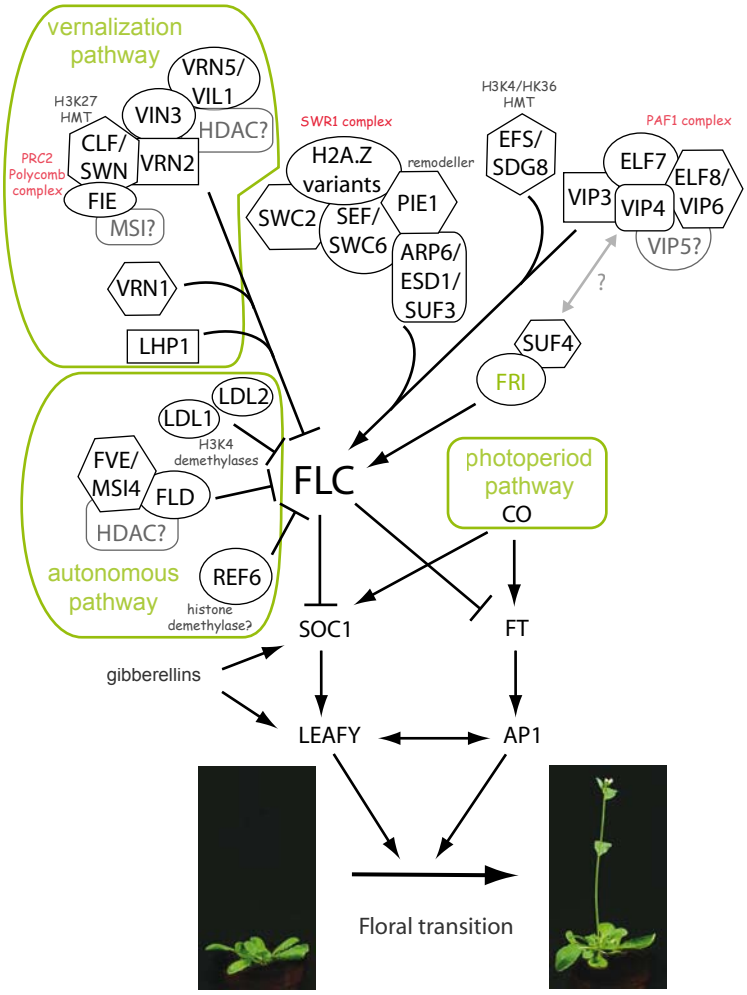


(A) Genomic imprinting in mammals involves erasure of the imprints in the primordial germ cells (PGCs) and subsequently acquisition of allele-specific imprints (blue square) during gametogenesis. This is maintained in the embryo, placenta and the adult. Therefore imprinting is an essential part of the mammalian life cycle and is an active process. (B) Genomic imprinting in plants is default (blue squares) and erasure of the imprint only takes place in the central cell by DEMETER, which contributes to the endosperm and is not involved in the plant life cycle.

repression by the autonomous pathway and results in *FLC* activation. SUF4, a putative zinc-finger-containing transcription factor, binds FRI and the *FLC* promoter and is responsible for H3K4me3 (Kim et al., 2006; Kim and Michaels, 2006).

Also, the PAF1 complex, consisting of ELF7, ELF8/VIP6, VIP3, VIP4 and possibly VIP5 in *Arabidopsis*, is necessary for H3K4 trimethylation of *FLC* (He et al., 2004; Oh et al., 2004; Zhang and van Nocker, 2002). In yeast, this complex is associated with RNA Polymerase II and recruits HMTs SET1 and SET2, which are responsible for H3K4me3 and H3K36me3, respectively. Two conflicting reports state that mutation of the putative *Arabidopsis* HMT *EFS*/*SDG8* reduces either H3K4me2 or H3K36me2 at *FLC* chromatin (Kim et al., 2005; Zhao et al., 2005). PIE1, an ISWI chromatin remodeller, activates *FLC* expression (Noh and Amasino, 2003). In yeast, the related ISW1p binds preferentially to H3K4me2/3 chromatin. Therefore, PIE1 might be recruited to H3K4 methylated by a SET1 homologue and remodel *FLC* chromatin to increase gene expression. Chromatin remodellers are found in large multiprotein complexes, like the SWR1 complex in yeast. The yeast SWR1 complex is involved in the exchange of H2A with histone variant H2A.Z, which potentiates optimal transcriptional activation. Recently, evidence for a

Figure 14 Pathways and chromatin factors regulating flowering time in *Arabidopsiss*



The autonomous pathway, the vernalization pathway, the PAF1 complex pathway and FRI converge on the floral repressor *FLC*. FLC and the photoperiod pathway regulate the flowering-time integrators, *SOC1* and *FT*. These activate floral-meristem identity genes, *LEAFY* and *AP1*, which promote the floral transition. Gibberellins regulate *SOC1* and *LEAFY*. FRI interacts with SUF4 and in this way might recruit the PAF1 complex to the *FLC* promoter. Chromatin complexes and their enzymatic activity are depicted. HMT, histone methyltransferase; HDAC, histone deacetylase.

SWR1 complex in *Arabidopsis* was found, consisting of PIE1, ARP6/ ESD1/SUF3, SEF/ SWC6, and SWC2 (Choi et al., 2005; Deal et al., 2005; March-Diaz et al., 2007; Martin-Trillo et al., 2006). The *Arabidopsis* SWR1 complex associates with *Arabidopsis* H2A.Z variants and was suggested to poise *FLC* for activation (Choi et al., 2007; Deal et al., 2007; March-Diaz et al., 2007). H3K4 trimethylation by the PAF1 complex is dependent on the presence of FRI and SUF4, and vice versa. It was suggested that SUF4 binds FRI and the *FLC* promoter and might recruit the PAF1 complex (Kim et al., 2006; Kim and Michaels, 2006). However, this hypothesis still awaits confirmation.

Finally, prolonged exposure to cold (vernalization) promotes flowering by permanent repression of *FLC*, which is preserved through mitotic cell divisions. During vernalization, H3K9me2 and H3K27me2 are increased around the *FLC* promoter. The chromatin binding protein VIN3 is expressed transiently during prolonged cold exposure, binds *FLC* through its PHD domain and is necessary for the initial repression of *FLC* (Sung and Amasino, 2004). Subsequently, VRN1 and VRN2 are required to maintain the repressed state of *FLC*. VRN1 encodes a DNA binding protein, which binds around the *FLC* promoter and is responsible for the H3K9me2 marks (Bastow et al., 2004). VRN2 is homologous to SU(Z)12, a PRC2 complex protein. In mammals and flies, the PRC2 complex was shown to possess H3K27 and possibly H3K9 methyl-transferase activity. Similarly, both H3K9me2 and H3K27me2 are reduced in *vrn2* mutants (Bastow et al., 2004; Gendall et al., 2001; Sung and Amasino, 2004). The VRN2 PRC2 complex consists of CLF, SWN and FIE (Wood et al., 2006). MSI1 is probably not a component of this complex, since it is involved in regulation of flowering time independent of *FLC*. Possibly another MSI homologue (MSI2-5) is present in the complex (Bouveret et al., 2006). In *Drosophila*, a protein containing two PHD fingers is responsible for recruitment of a HDAC to the complex. Similarly, the VRN2 complex contains VIN3 and might include an unknown histone deacetylase responsible for the histone deacetylation at *FLC* (Wood et al., 2006). VIN3 interacts with another PHD finger protein, VRN5/VIL1, which acts at *FLC* similar to VIN3 (Greb et al., 2007; Sung et al., 2006b). The *Arabidopsis* homologue of HP1, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), was also found to be involved in the maintenance of *FLC* repression. LHP1 is increasingly associated with *FLC* during vernalization and H3K9me2 is lost in *lhp1* mutants (Mylne et al., 2006; Sung et al., 2006a). This is in contrast with the recent finding that LHP1 specifically colocalizes with H3K27me3 (Turck et al., 2007; Zhang et al., 2007), therefore loss of H3K9me2 might be secondary. In contrast to mammals, a

PRC1 complex is absent in plants. *Arabidopsis* LHP1 could play a role in the maintenance of repression, similar to the mammalian PRC1 complex. (Sung et al., 2006a). Initial repression is probably mediated by other factors (possibly histone deacetylation) during cold exposure, while the VRN2 PRC2 complex and LHP1 maintain *FLC* repression thereafter (Sung et al., 2006a).

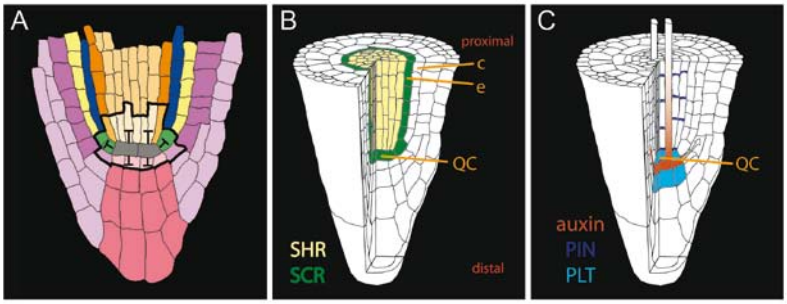
Taken together, many different chromatin factors act in several flowering time regulating pathways to influence *FLC* expression by modifying *FLC* chromatin around its promoter (Figure 14). Chromatin provides a way to regulate heritable multi-step regulation of gene expression. The autonomous pathway constitutively represses *FLC* by histone deacetylation and H3K4 demethylation to promote flowering. However, in the presence of the FRI pathway and the PAF1 complex pathway flowering is prevented by activating *FLC* expression using H3K4 dimethylation and probably chromatin remodelling. The vernalization pathway overrules the activation by the FRI pathway and PAF1 complex pathway, and represses *FLC* permanently by deacetylation and dimethylation of H3K9 and H3K27 through the PRC2 complex together with LHP1. This results in a stable epigenetic repression of *FLC* and induction of flowering.

2.2.3 *Arabidopsis* stem cells

Similar to animals, plant stem cells are regulated by extrinsic signals from their niche (reviewed by Scheres, 2007; Singh and Bhalla, 2006; Stahl and Simon, 2005; Vernoux and Benfey, 2005; Williams and Fletcher, 2005). Therefore, equivalent mechanisms may be present to control stem cells although multicellularity arose independently in both kingdoms. Post-embryonically, plant stem cells are located and maintained at two sites, the shoot apical meristem (SAM) and the root meristem (RM). Plant specific transcription factors are involved in stem cell specification, illustrating the separate evolution of stem cell patterning in both kingdoms.

The SAM contains stem cells, which produce all above-ground tissues. These stem cells are maintained at the population level and are controlled through two parallel pathways. The initiation and maintenance of the SAM and the suppression of differentiation is regulated by the homeodomain transcription factor SHOOT MERISTEMLESS (STM). In addition, stem cell maintenance is regulated by homeodomain transcription factor WUSCHEL (WUS) and the CLAVATA (CLV) receptor kinase pathway. *WUS* is expressed in the organizing centre and induces the expression

Figure 15 Root tip with several cell types and the pathways that position the stem cell niche



(A) Different cell types in the root tip derive from the stem cell niche. Signals from the QC or organizer centre maintain the surrounding stem cells. Black line outlines stem cell niche. Grey, quiescent centre (QC); pink, columella root cap; light purple, lateral root cap; dark purple, epidermis; green, cortex/endodermis stem cell; yellow, cortex; blue, endodermis; light and dark orange, vascular bundle with pericycle (dark orange). (B-C) Two parallel pathway position the root stem cell niche. The SHR/SCR pathway (B) and the PLT pathway (C). (B) *SHR* is expressed in the vasculature and moves into the endodermis (e) where it activates *SCR* expression. SCR is required for the asymmetric division of the cortex/endodermis stem cell resulting in endodermis and cortex (c). (C) The phytohormone auxin accumulates at the position of the stem cell niche, which is achieved by the polar localization of PIN proteins (putative auxin efflux carriers) in the plasmamembrane. The auxin maximum regulates the expression of *PLT1* and *PLT2*.

of the *CLV3* ligand in the overlying stem cells. CLV3 binds the CLV1-CLV2 receptor kinase complex, which represses the expression of *WUS*. This negative feedback loop regulates the size of the stem cell pool (reviewed by Stahl and Simon, 2005; Vernoux and Benfey, 2005; Williams and Fletcher, 2005). Chromatin factors are found to be involved in stem cell maintenance in plants. The SNF2 chromatin remodelling factor SPLAYED (SYD) maintains the SAM during the reproductive stage through the WUS pathway. The SAM defect of *syd* is partially rescued by *clv1*, suggesting that SYD does not regulate *WUS* through the CLV pathway. SYD directly interacts with the *WUS* promoter, regulates *WUS* transcription and thereby promotes stem cell maintenance in the shoot (Kwon et al., 2005). This is similar to the role of remodelling factors in stem cell maintenance in the *Drosophila* ovary (Xi and Xie, 2005) and in mammalian neural stem/progenitor cells (see section 2.1.3) (Lessard et al., 2007). This implies that chromatin factors are involved in stem cell maintenance in plants like in animals.

The RM contains stem cells, which produce all under-ground tissues. These stem cells surround and are physically linked to the organising centre or quiescent centre (QC), which is required for their maintenance (Figure 15A) (van den Berg et al., 1995). There are two pools of stem cells present, which produce daughter cells by asymmetric divisions and have different characteristics. The daughters deriving from the distal stem cells do not divide and differentiate into the columella cell type (Figure 15A), which enables the root to perceive gravity and is found in the root cap. The proximal stem cell daughters form a transit amplifying cell population in which a few extra rounds of cell division take place. These cells contribute to the growth of the root by expansion and differentiate into several cell types (vascular, epidermal, cortex and endodermis cells) (Figure 15A). The proximal stem cells also produce lateral root cap cells, which form the root cap together with the columella cells. The cap protects the stem cell niche and is thought to aid root growth in soil by detaching cells.

The position of the root stem cell niche is controlled through two parallel plant specific pathways (Figure 15). The stem cell niche is positioned in the apical-basal direction through the accumulation of the phytohormone auxin, which is achieved by the polar localization of PIN proteins (putative auxin efflux carriers) in the plasmamembrane (Blilou et al., 2005). This auxin maximum regulates the expression of the AP2 domain transcription factors *PLETHORA1* (*PLT1*) and *PLT2*, which are redundantly required for stem cell maintenance (Figure 15C) (Aida et al., 2004). In turn, the PLT proteins regulate the expression of *PIN* genes, revealing a feedback loop (Blilou et al., 2005). The stem cell niche is controlled in the radial direction through the GRAS family transcription factors SHORTROOT (SHR) and SCARECROW (SCR). *SHR* is expressed in the vascular tissue and moves into the outer layer (endodermis and QC), where it induces *SCR* expression (Figure 15B). SCR is required cell-autonomously in the QC to maintain the surrounding stem cells (Heidstra et al., 2004; Helariutta et al., 2000; Nakajima et al., 2001; Sabatini et al., 2003). The position of the stem cell niche is defined by the overlap between the highest *PLT* and *SCR* expression (Aida et al., 2004). The combination of key transcription factor pathways to program stem cells resembles the combined action of pluripotent transcription factor Oct4, Nanog and Sox2 in animal stem cells (see section 2.1.3) (Johnson et al., 2006; Niwa, 2007a).

SCR appears to inhibit RETINOBLASTOMA RELATED (RBR) activity in the QC or in the stem cells (Wildwater et al., 2005). RBR is involved in stem cell maintenance, since

overexpression of *RBR* causes stem cell loss and reduction of *RBR* results in more stem cells. In addition, reduction of *RBR* is sufficient to rescue stem cell defects in the *scr* mutant. Stem cell loss upon ablation of the QC shows that at all times a functional QC is required. Cell cycle progression is not influenced by reduction of *RBR*, suggesting that differentiation is affected. It is not clear yet whether RBR acts in the QC or in the stem cells (Wildwater et al., 2005). In this respect, it is interesting to note that RB was recently shown to be essential in the mouse bone marrow microenvironment/niche, but not in the stem cells (Walkley et al., 2007). Differentiation is promoted by RB through its association with transcription factors in mammals (reviewed by Korenjak and Brehm, 2005; Skapek et al., 2006). In addition, RB is known to be associated with many chromatin factors, which mediate repression of RB targets (reviewed by Frolov and Dyson, 2004; Giacinti and Giordano, 2006). Possibly, similar mechanisms might act in plant stem cells.

Another chromatin factor implicated in both SAM and RM maintenance is the *Arabidopsis* chromatin assembly factor-1 (CAF-1) complex. The mammalian CAF-1 complex subunits p150 and p60 are encoded by the *Arabidopsis FAS1* and *FAS2* genes, which are (among other things) involved in nucleosome assembly and stable maintenance of epigenetic states (Costa and Shaw, 2006; Kaya et al., 2001; Ramirez-Parra and Gutierrez, 2007a; Ramirez-Parra and Gutierrez, 2007b; Schonrock et al., 2006b). The SAM in *fas* mutants is broader and flatter, the organization is disrupted and *WUS* expression is expanded. In the RM, stem cells are lost in *fas* mutants and *SCR* expression is not maintained properly (Kaya et al., 2001). Therefore, the *Arabidopsis* CAF-1 complex is thought to be involved in the stable maintenance of key developmental genes through correct chromatin formation. The *Arabidopsis* CAF-1 complex was shown to be involved in the chromatin conformation at the homeobox gene *GLABRA2 (GL2)*. GL2 is an atrichoblast cell fate determinant, which is expressed in non-hair root cells (atrichoblasts) and is repressed in hair root cells (trichoblasts). Accordingly, the chromatin conformation specifically at the *GL2* locus is 'open' in atrichoblasts and 'closed in trichoblasts. In *fas* mutants, the chromatin conformation at the *GL2* locus is open irrespective of the cell type and *GL2* is ectopically expressed (Costa and Shaw, 2006). Furthermore, in *fas* mutants, the heterochromatin fraction is reduced without affecting heterochromatin specific epigenetic marks (DNA methylation and H3K9me2) (Kirik et al., 2006; Schonrock et al., 2006b). This suggests that the CAF-1 complex is involved in heterochromatin compaction. Similarly, the largest

subunit of CAF-1 (p150) was found to be essential for the assembly of the heterochromatin organisation in mouse embryonic stem cells and epigenetic marks at pericentric heterochromatin were affected (see section 2.1.3) (Houlard et al., 2006). This indicates similarities between chromatin organisation in animal and plant stem cells. However, histone modifications (DNA methylation, H3K9me2, H3 and H4 acetylation) are not affected globally in *fas* mutants (Ramirez-Parra and Gutierrez, 2007a; Schonrock et al., 2006b). Only a small fraction of heterochromatic genes is upregulated in *fas* mutants and these were mainly expressed during late S phase (Schonrock et al., 2006b). In addition, these genes (but not their neighbouring genes) contained less H3K9me2 and more H3 and H4 acetylation (Ramirez-Parra and Gutierrez, 2007a). This suggests that the CAF-1 complex ensures the proper epigenetic inheritance of specific genes in *Arabidopsis*, while in animals the CAF-1 complex has a more general role in heterochromatin formation. In human and mouse cells, depletion of CAF-1 results in S phase arrest and eventually cell death (Hoek and Stillman, 2003; Houlard et al., 2006; Nabatiyan and Krude, 2004). In *Arabidopsis*, deletion of *FAS1* or *FAS2* causes an increase in ploidy levels in seedlings and leaves (Endo et al., 2006; Exner et al., 2006; Kirik et al., 2006; Ramirez-Parra and Gutierrez, 2007a). This suggests that instead of cell cycle arrest, like in animals, endoreplication is enhanced in plants to overcome the G2 checkpoint.

Taken together, animal and plant stem cells and their organizers have a strikingly similar organisation, although multicellularity arose independently in both kingdoms (Scheres, 2007). Therefore, transcription factors, RB and chromatin factors (like Polycomb complexes, remodellers and the CAF-1 complex) are likely candidates to serve as general stem cell control factors.

Aim of this thesis

Chromatin factors are required to obtain a special chromatin state in mammalian stem cells (**Chapter 1**). The role of chromatin factors in plant stem cell control is just starting to be revealed. To identify the importance of the chromatin state for the root stem niche in *Arabidopsis*, we performed a genome-wide survey of chromatin factor mutants that display phenotypes in stem cells or organizer cells. In addition, we investigated the relationship of these chromatin factors with existing stem cell regulatory pathways.

In **Chapter 2**, we describe the role of HAT GCN5 in root stem cell niche maintenance. Both GCN5 and its associated factor ADA2b regulate expression levels of the stem cell transcription factors *PLETHORA1 (PLT1)* and *PLT2*, and act in the PLT pathway. Overexpression of *PLT2* rescues the stem cell niche defect of *gcn5* mutants. Interestingly, ADA2b is not involved in stem cell niche maintenance, but mediates proliferation of the transit amplifying cells directly.

In **Chapter 3**, we investigate the role of the *Arabidopsis* CAF-1 complex in root stem cell niche maintenance. We show that the CAF-1 complex and RETINOBLASTOMA RELATED (RBR) repress synergistically root stem cell proliferation through regulation of stem cell niche transcription factors and promote correct differentiation. Acute reduction of the CAF-1 complex or RBR and treatments with histone deacetylase inhibitor TSA indicate that stem cell loss and overproliferation are caused by an accumulation of epigenetic defects.

In **Chapter 4**, we examine the role of PRC2 Polycomb complexes in root stem cell niche maintenance. We show that SWINGER (SWN) and CURLY LEAF (CLF) are redundantly required to maintain the stem cells and expression of the root stem cell transcription factor *SCARECROW (SCR)*. In addition, SWN and CLF influence the transit amplifying cells together with RBR, revealing a novel function for RBR.

In **Chapter 5**, the results from Chapter 2, 3 and 4 are discussed in light of recent insights into the specialized chromatin state in stem cells.

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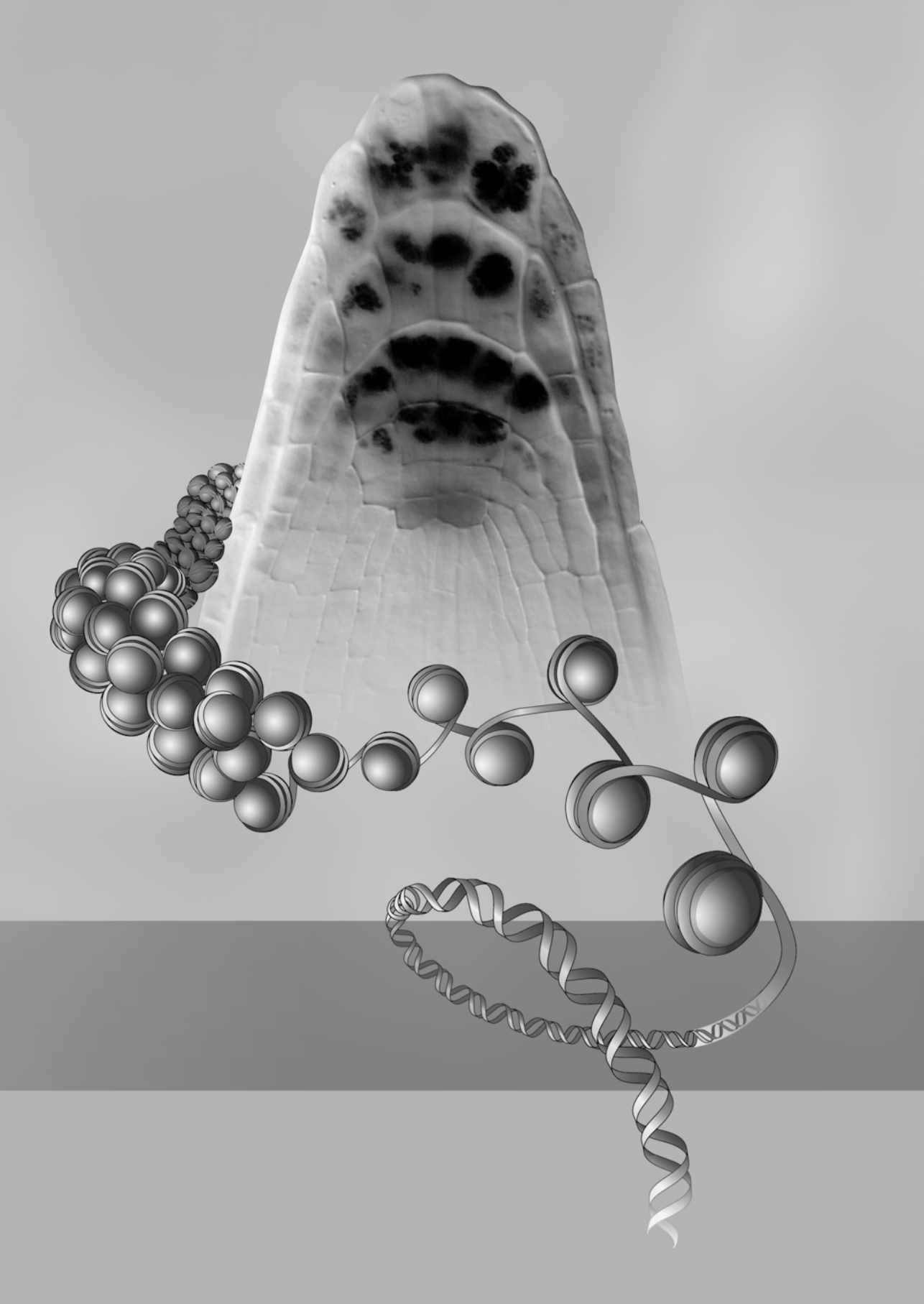


CHAPTER 2

Arabidopsis GCN5 and ADA2b have overlapping yet distinct functions in the PLT pathway regulating stem cell niche maintenance and proliferation of transit amplifying cells

Noor Kornet and Ben Scheres

Summary

Histone acetylation is known to facilitate gene transcription and plays an important role in developmental processes. Here, we show that histone acetyltransferase GCN5 is essential for root stem cell niche maintenance, specifically regulates expression of the stem cell transcription factor *PLETHORA (PLT)*, and acts in the PLT pathway based on genetic evidence. Overexpression of *PLT2* rescues the stem cell niche defect of *gcn5* mutants, indicating that GCN5 regulates *PLT* expression and thereby aids in specifying the root stem cell niche. The GCN5 associated factor ADA2b regulates *PLT* expression and genetic interactions position it in the PLT pathway, similar to GCN5. However, stem cell niche maintenance is not affected in the *ada2b* mutant. Instead, ADA2b mediates meristem size and proliferation of the transit amplifying cells directly. Our data suggest that GCN5 and ADA2b have overlapping yet distinct roles in the root, affecting the stem cell niche and transit amplifying cells, respectively.

Introduction

Chromatin modifications play an important role in gene transcription. Both the nucleosomal histone core and the histone tails can be post-translationally modified by acetylation, methylation, phosphorylation, ubiquitination and many other modifications. Histone acetylation is thought to enhance the accessibility of the DNA and facilitate transcription (reviewed by Lee and Workman, 2007; Shahbazian and Grunstein, 2007). Histone acetyltransferases (HATs) are found in coactivator complexes and histone deacetylases (HDACs) are found in repressor complexes. The balance between HAT and HDAC activities is thought to determine the level of gene expression.

There are four HAT families and GCN5, a member of the GNAT family, is the best studied HAT in yeast and mammals (reviewed by Baker and Grant, 2007; Lee and Workman, 2007; Nagy and Tora, 2007; Shahbazian and Grunstein, 2007). In *Arabidopsis*, similar HAT families have been found and the single *Arabidopsis* GCN5 homologue has been best characterized (Chen and Tian, 2007; Pandey et al., 2002). Similar to yeast and mammals, *Arabidopsis* GCN5 contains a bromodomain, has been shown to acetylate H3 *in vitro* (Mao et al., 2006; Stockinger et al., 2001) and global H3 acetylation is reduced in *gcn5* mutants (Bertrand et al., 2003). More specifically, H3K14 and H3K27 acetylation are reduced at defined loci in *gcn5* mutants (Benhamed et al., 2006).

Yeast GCN5 is found in the SAGA and ADA complexes together with ADA2 and similar complexes exist in human and *Drosophila* (Nagy and Tora, 2007). Also, in *Arabidopsis*, GCN5 interacts *in vitro* with the two homologues ADA2a and ADA2b (Mao et al., 2006; Stockinger et al., 2001). *Arabidopsis* ADA2b enhances the HAT activity of GCN5 (Mao et al., 2006), which is also found in yeast. A subset of genes is regulated by the *Arabidopsis* GCN5 complex, since expression of 5% of investigated 8200 genes is changed in *gcn5* and *ada2b* mutant leaves (Vlachonasios et al., 2003).

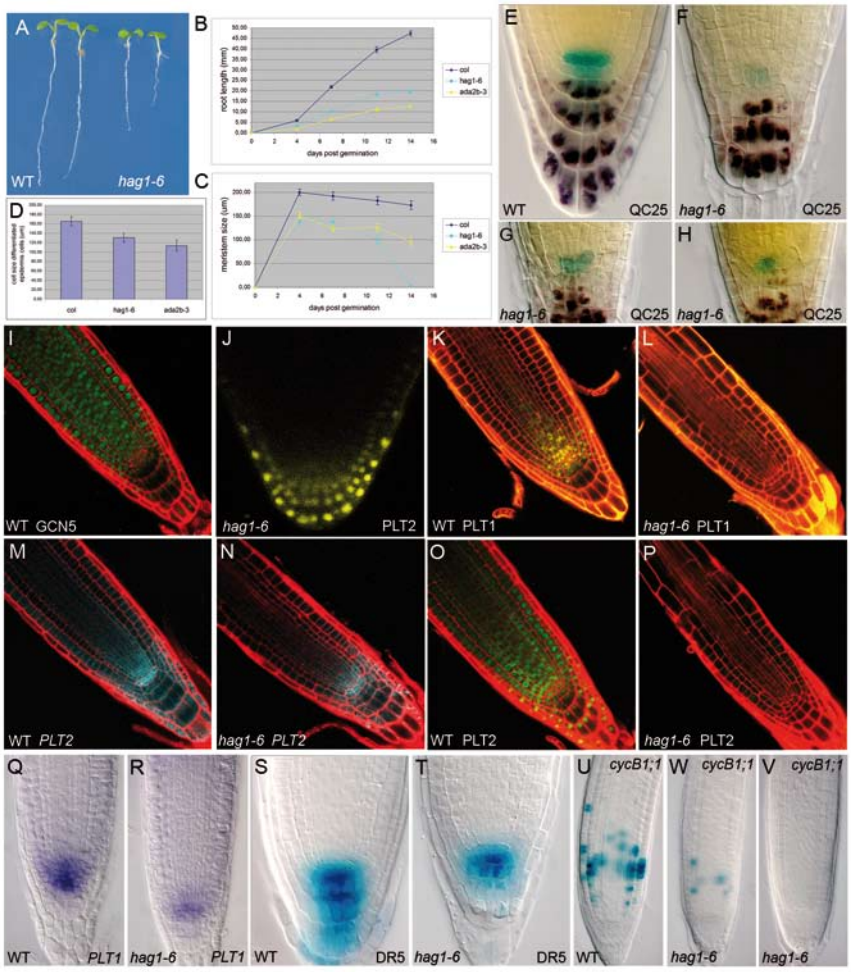
The *Arabidopsis* GCN5 complex has been implicated in developmental processes and the response to environmental stress. Mutation of *GCN5* and *ADA2b* causes pleiotropic defects in shoot and root (Benhamed et al., 2006; Bertrand et al., 2003; Vlachonasios et al., 2003). GCN5 was found in a suppressor screen of the *topless* (*tpl*) mutation. TPL acts as a repressor of root identity in the shoot during embryogenesis.

Mutation of *GCN5* restores the expression of *WUS* in *tpl* mutants and rescues shoot formation (Long et al., 2006). In addition, the flower meristem defects of *gcn5* mutants are probably caused by ectopic expression of homeodomain protein WUS (Bertrand et al., 2003). It is unclear whether *WUS* is a direct target of GCN5. There are two examples of the role of the GCN5 complex in transcriptional regulation due to environmental stress. First, GCN5 and ADA2b bind *in vitro* to the transcription factor CBF1, which is involved in cold-regulated (COR) gene expression (Mao et al., 2006; Stockinger et al., 2001; Vlachonasios et al., 2003). Second, GCN5 is involved in light-regulated gene expression (Benhamed et al., 2006).

gcn5 and *ada2b* mutants have smaller roots (Vlachonasios et al., 2003). Root growth is maintained by the organising centre or quiescent centre (QC) and its surrounding stem cells (van den Berg et al., 1995). The stem cell niche in the root is specified by two parallel transcription factor pathways: the PLETHORA (PLT) pathway (Aida et al., 2004; Blilou et al., 2005) and the SHORT-ROOT (SHR)/ SCARECROW (SCR)/ RETINO-BLASTOMA RELATED (RBR) pathway (Helariutta et al., 2000; Nakajima et al., 2001; Sabatini et al., 2003; Wildwater et al., 2005).

Here, we investigated in detail the role of GCN5 and ADA2b in root development and how these chromatin factors relate to the known transcription factors involved in root patterning. We show that GCN5 and ADA2b act specifically through the PLT pathway and increase *PLT1* and *PLT2* expression levels. GCN5 is involved in the maintenance of the root stem cell niche. Overexpression of *PLT2* rescues the stem cell niche defect of *gcn5* mutants, indicating that GCN5 affects root stem cell niche maintenance mainly through regulation of *PLT* expression. ADA2b regulates *PLT1*, *PLT2* and *cycB1;1* expression similar to GCN5. However, the root stem cell niche is not lost in *ada2b* mutants despite low PLT levels, but rather meristem size and the transit amplifying cells are affected. This reveals that GCN5 and ADA2b have overlapping yet distinct roles in the PLT pathway. The presence of a single *ada2b* allele impedes the enlargement of the meristem due to *PLT2* overexpression, indicating that ADA2b controls the proliferation potential of the transit amplifying cells.

Figure 1 GCN5 is required for stem cell niche maintenance, *PLT1, PLT2* and *cycB1;1* expression



(A) Phenotype of wildtype and *hag1-6* mutant, 7 dpg. (B-D) Root length (B) meristem size (C) and size of differentiated epidermis cells 7 dpg (D) of *hag1-6* and *ada2b-3* seedlings compared to wildtype, error bars indicate 95% confidence interval. (E-H) QC25 expression and starch granules in wildtype (E) and *hag1-6* (F-H),7 dpg. (I) *pGCN5::GCN5:GFP*, 8 dpg. (J) Expression of *pPLT2::PLT2:YFP* in *hag1-6* embryos. (K-L) Expression of *pPLT1::PLT1:YFP* in wildtype (K) and *hag1-6* (L), 4 dpg. (M-N) Expression of *pPLT2::CFP* in wildtype (M) and *hag1-6* (N), 7 dpg. (O-P) Expression of *pPLT2::PLT2:GFP* in wildtype (O) and *hag1-6* (P), 4 dpg. (Q-R) Whole-mount in situ hybridisation with *PLT1* probe in wildtype (Q) and *hag1-6* (R) seedlings, 3 dpg. (S-T) Expression of *DR5::GUS* in wildtype (S) and *hag1-6* (T), 7 dpg. (U-V) Expression of *cycB1;1:GUS* in wildtype (U) and *hag1-6* (W-V), 5 dpg.

Results

GCN5 regulates stem cell niche maintenance and *PLT* expression levels

To investigate the role of GCN5 in root development, we obtained T-DNA insertions in the 9th intron (SALK_048427) and the 1st intron (SALK_150784) of the *GCN5* gene. These alleles were described previously as *hag1-5* and *hag1-6*, respectively (Long et al., 2006). The *hag1-5* allele contains a bromodomain deletion, while the *hag1-6* allele disrupts both the acetyltransferase domain and the bromodomain. Disruption of *GCN5* causes pleiotropic defects in the shoot as described before (Benhamed et al., 2006; Bertrand et al., 2003; Vlachonasios et al., 2003) and shorter roots (Figure 1A,B) (Vlachonasios et al., 2003). *hag1-5* seedlings show a less severe phenotype and mutant plants are able to produce viable seeds. *hag1-6* mutant seedlings possess a more severe phenotype, shorter roots and meristem size compared to wildtype (Figure 1A,B,C). The root meristem of *hag1-6* mutants is not maintained and differentiates at 14 dpg (Figure 1C). The cell size of differentiated epidermis cells is significantly smaller than wildtype (Figure 1D; *t*-test, $P = 6.5\ 10^{-6}$). *hag1-6* mutant plants are infertile, therefore they were maintained as a heterozygous population. Digital in situ hybridisation (Birnbaum et al., 2003) indicates that *GCN5* is ubiquitously expressed in all cells of the root, which we confirm by a *GCN5* translational fusion (Figure 1I).

To assess whether the stem cell niche is affected in the *hag1-6* mutant, a QC specific marker (QC25) was used in combination with a columella differentiation marker (starch granules). In wildtype, a layer of columella stem cells is present between the QC and differentiated columella cells (which contain starch granules) (Figure 1E). In contrast, *hag1-6* mutants show starch granules next to the QC (20%, n=30) (Figure 1G,H, Supplementary Table 1) or in the QC (30%, n=30) (Figure 1F,G, Supplementary Table1). In addition, QC25 is completely absent (13,3%, n=30) (Supplementary Table1) or absent from some QC cells (30 %, n=30) (Figure 1H, Supplementary Table1). This indicates a defect in QC function and columella stem cell loss, leading to root meristem differentiation. In addition, the number of differentiated columella layers is significantly smaller (Figure 1E-F; *t*-test, $P = 2.0\ 10^{-3}$).

The position of the stem cell niche is defined by the overlap between the highest *PLT* and *SCR* expression (Aida et al., 2004). In addition, *SHR* is expressed in the vascular

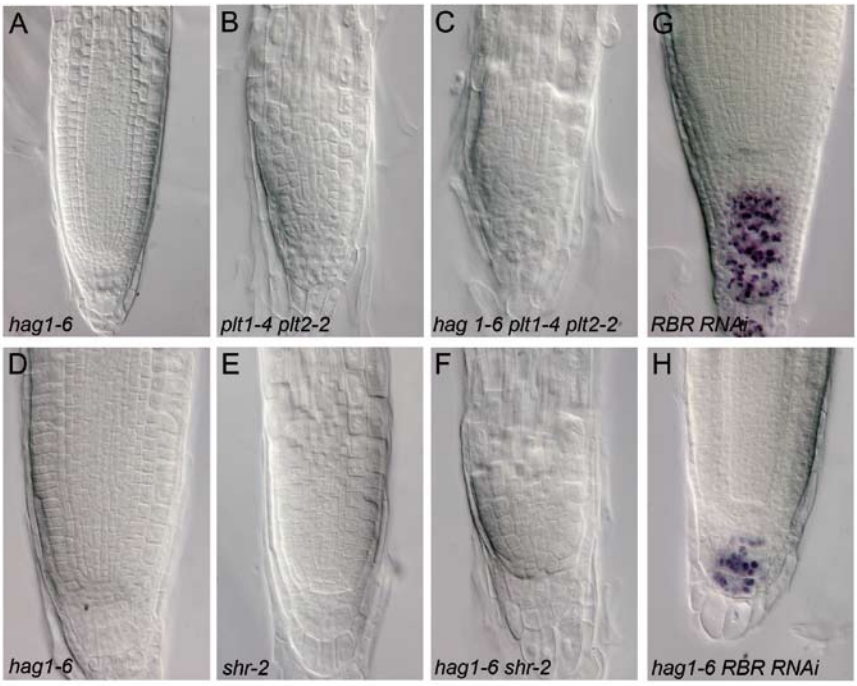
tissue and moves into the outer layer (endodermis and QC), where it induces *SCR* expression (Helariutta et al., 2000; Nakajima et al., 2001). To determine whether *SCR* or *SHR* expression is affected in the *hag1-6* mutant, a *pSCR::GFP* transcriptional and a *pSHR::GFP:SHR* translational fusion (Nakajima et al., 2001) were used. Neither expression of *SCR* (Supplementary Figure 1B) nor localization of SHR (Supplementary Figure 1D) is affected in *hag1-6* mutants compared to wildtype (Supplementary Figure 1A,C). In contrast, in situ hybridization with a *PLT1* probe revealed that expression of *PLT1* was reduced in *hag1-6* mutants (Figure 1R) compared to wildtype (Figure 1Q). Using a *PLT1* translational fusion (Galinha et al., 2007), PLT1 protein levels are consistently downregulated in *hag1-6* mutants (Figure 1K,L, Supplementary Figure 1E). Similarly, transcriptional and translational fusions of *PLT2* (Galinha et al., 2007) are severely reduced in *hag1-6* mutants (Figure 1M-P, Supplementary Figure 1F). In *hag1-6* embryos, the expression of the translational fusion of *PLT2* is not affected (Figure 1J), indicating that GCN5 affects *PLT2* expression post-embryonically.

PLT proteins regulate the PIN proteins (putative auxin efflux carriers) and vice versa, thereby fine-tuning the position of the auxin maximum (Blilou et al., 2005). Therefore auxin distribution was analysed using the *DR5::GUS* construct (Ulmasov et al., 1997). The position of the auxin maximum is not changed in *hag1-6* mutants (Figure 1S,T), indicating that GCN5 does not regulate *PLT1* and *PLT2* expression through modulation of auxin perception or distribution. The expression of *cyclinB1;1:GUS* (marker for the G2/M phase of the cell cycle) (Colon-Carmona et al., 1999) is reduced or even absent in *hag1-6* mutants (Figure 1U-V. Supplementary Figure 1G), similar to *plt1-4 plt2-2* double mutants (Aida et al., 2004). This suggests that the population of dividing cells in the proximal meristem of *hag1-6* mutants is smaller.

GCN5 acts in the PLT pathway

To further assess whether GCN5 acts through the PLT pathway or the SHR/SCR/RBR pathway, different combinations with null mutants were made. The *hag1-6 shr-2* double mutant seedlings possess shorter roots and smaller meristem size at 7 dpg compared to both single mutants (Figure 2D-F, Supplementary Figure 2B,E). This indicates that GCN5 acts in a pathway parallel to SHR. RBR acts genetically downstream of the SHR target SCR and reduction of *RBR* leads to additional columella stem cell layers (Figure 2G) (Wildwater et al., 2005). However, reduction of *RBR* is not able to rescue meristem

Figure 2 GCN5 acts in the PLT pathway



(A-C) Root tip of *hag1-6* (A), *plt1-4 plt2-2* (B) and *hag1-6 plt1-4 plt2-2* (C), 4 dpg (Note: scale of *hag1-6* meristem is different). (D-F) Root tip of *hag1-6* (D), *shr-2* (E) and *hag1-6 shr-2* (F), 7 dpg. (G-H) Root tip of *RBR RNAi* (G) and *hag1-6 RBR RNAi*, 6 dpg (H).

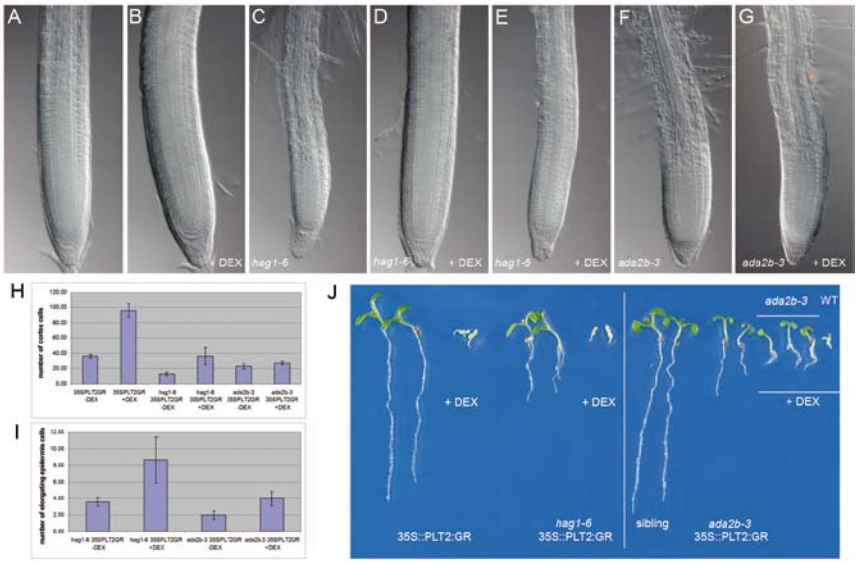
size or root length of *hag1-6* mutants (Supplementary Figure 2C), although additional columella stem cell layers can be transiently observed (Figure 2H, Supplementary Figure 2F). Together, these data indicate that GCN5 acts independent of the SHR/SCR/RBR pathway.

To assess whether GCN5 acts in the PLT pathway, *hag1-6* plants were crossed to *plt1 plt2* double mutant plants (Aida et al., 2004). *hag1-6 plt1-4 plt2-2* triple mutants show similar root length compared to *plt1-4 plt2-2* double mutants (Supplementary Figure 2A). In addition, *hag1-6 plt1-4 plt2-2* triple mutants possess a similar meristem size and number of cortex cells as *plt1-4 plt2-2* double mutants (Figure 2A-C, Supplementary Figure 2D). These data confirm that GCN5 acts in the PLT pathway.

The *gcn5* stem cell niche defect can be rescued by overexpression of *PLT2*

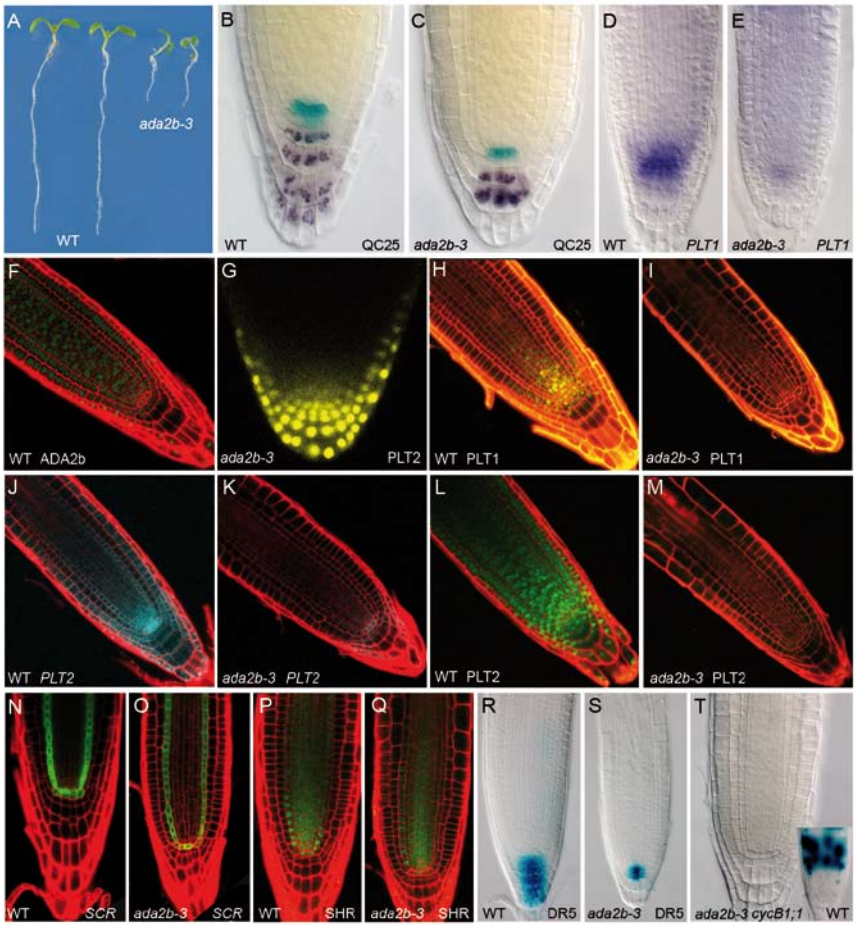
To determine whether overexpression of *PLT* genes can bypass the stem cell niche defects in *hag1-6* mutants, a *35S::PLT2:GR* construct was introduced (Galinha et al., 2007). When dexamethasone is applied to *35S::PLT2:GR* seedlings, proximal meristem size and number of cortex cells are increased substantially as reported previously (Figure 3A,B,H). In the *hag1-6* background, meristem size and number of cortex cells in the proximal meristem are significantly increased when dexamethasone is added (Figure 3C-E,H; *t*-test, $P = 1.5\ 10^{-3}$). In addition, the size of the elongation zone and the number of epidermis cells in the elongation zone of *hag1-6* mutants are significantly increased due to overexpression of *PLT2* (Figure 3I; *t*-test, $P = 1.5\ 10^{-2}$). At 13 dpg,

Figure 3 Overexpression of *PLT2* in *hag1-6* and *ada2b-3* mutants



(A-G) Overexpression of *PLT2*, without (A,C,E) and 3 days after 2 μM dexamethasone (DEX) treatment (B,D,E,G) of *35S::PLT2:GR* (A,B), *hag1-6 35S::PLT2:GR* (C,D,E) and *ada2b-3 35S::PLT2:GR* (F,G), 8 dpg. (H-I) Quantification number of cortex cells (H) and elongating epidermis cells (I) induced by *35S::PLT2:GR* in *hag1-6* and *ada2b-3*. Error bars indicate 95% confidence interval. (J) Overexpression of *PLT2* with application of dexamethasone (DEX) directly at germination in *hag1-6* and *ada2b-3* seedlings, 7 dpg.

Figure 4 ADA2b is required for *PLT1*, *PLT2* and *cycB1;1* expression



(A) Phenotype of wildtype and *ada2b-3* mutant, 7 dpg. (B-C) QC25 expression and starch granules in wildtype (B) and *ada2b-3* (C), 7 dpg. (D-E) Whole-mount in situ hybridisation with *PLT1* probe in wildtype (D) and *ada2b-3* seedlings (E), 3 dpg. (F) *pADA2b::ADA2b:GFP*, 6 dpg. (G) Expression of *pPLT2::PLT2:YFP* in *ada2b-3* embryos. (H-I) Expression of *pPLT1::PLT1:YFP* in wildtype (H) and *ada2b-3* (I), 4 dpg. (J-K) Expression of *pPLT2::CFP* in wildtype (J) and *ada2b-3* (K), 7 dpg. (L-M) Expression of *pPLT2::PLT2:GFP* in wildtype (L) and *ada2b-3* (M), 4 dpg. (N-O) Expression of *pSCR::GFP* in wildtype (N) and *ada2b-3* (O), 6 dpg. (P-Q) Expression of *pSHR::GFP:SHR* in wildtype (P) and *ada2b-3* (Q), 5 dpg. (R-S) Expression of *DR5::GUS* in wildtype (R) and *ada2b-3* (S), 7 dpg. (T) Expression of *cycB1;1:GUS* in *ada2b-3* (T), inset wildtype, 7 dpg.

47% (n=15) of the seedlings possess an enlarged meristem (data not shown), whereas 53% of the seedlings is differentiated around that time, similar to the *hag1-6* mutant

(Figure 1C). Together, these data show that ectopic expression of *PLT2* fully or transiently rescues the *hag1-6* defects in the stem cell niche and the elongation zone, and it confirms that GCN5 acts in the PLT pathway.

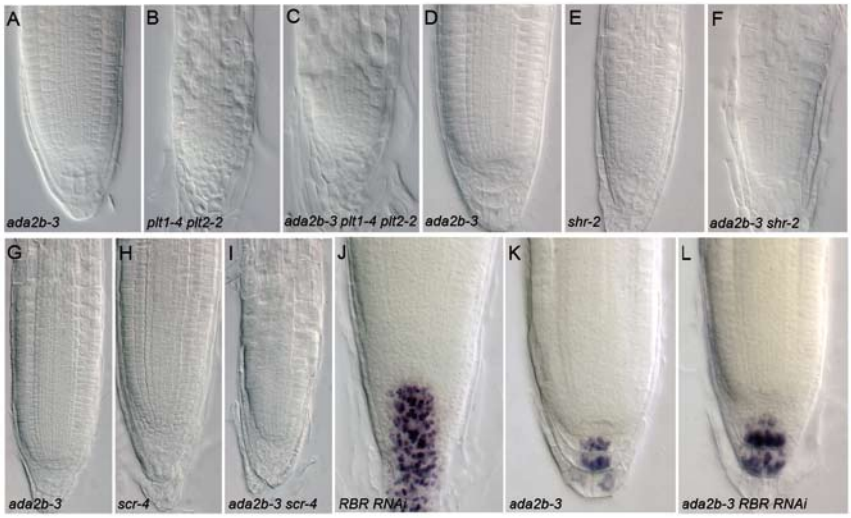
ADA2b regulates meristem size, *PLT* expression levels and proliferation of transit amplifying cells

The GCN5 coactivator complexes in yeast and mammals contain the ADA2 protein (reviewed by Nagy and Tora, 2007). Similarly, *Arabidopsis* ADA2a and ADA2b interact with GCN5 *in vitro* (Mao et al., 2006; Stockinger et al., 2001). To investigate the role of ADA2b in stem cell niche maintenance, we obtained T-DNA insertion lines in the 5th intron (*ada2b-1*) (Vlachonasios et al., 2003), and in the 9th exon of *ADA2b* (SALK_019407), hereafter called *ada2b-3*. *ada2b* mutants show pleiotropic defects in the shoot and shorter roots, as described previously (Figure 4A, 1B) (Sieberer et al., 2003; Vlachonasios et al., 2003). Root size of *ada2b-3* mutant seedlings is shorter compared to wildtype and *hag1-6* mutants (Figure 1B), whereas meristem size is smaller than wildtype but constant (Figure 1C). The cell size of differentiated epidermis cells in *ada2b-3* mutants is smaller than wildtype (Figure 1D; *t*-test, $P = 1.9\ 10^{-8}$). *ADA2b* is constitutively expressed in all cells of the root as shown by a translational fusion (Figure 4F), confirming the digital in situ hybridisation (Birnbaum et al., 2003).

A layer of columella stem cells is present in *ada2b-3* mutants between the QC and differentiated columella cells (Figure 4B,C). This indicates that, in contrast to *GCN5*, the stem cell niche is not affected by mutation of *ADA2b*. However, the number of differentiated columella layers is significantly reduced in *ada2b-3* mutants (Figure 4B-C; *t*-test, $P = 4.4\ 10^{-6}$) like in *hag1-6* mutants.

Expression of *SCR* transcript (Figure 4N,O) and localization of SHR protein (Figure 4P,Q) is not changed in *ada2b-3* mutants, and transcript and protein of *PLT1* and *PLT2* are reduced (Figure 4D-E, 4H-M, Supplementary Figure 3A,B). ADA2b affects *PLT2* expression post-embryonically, since *PLT2* is expressed in *ada2b-3* embryos (Figure 4G). While expression of *cyclinB1;1:GUS* is reduced in *hag1-6* mutants, its expression is completely absent in *ada2b-3* mutants (Figure 4T). The auxin maximum is not affected in *ada2b-3* mutants (Figure 4R,S), although auxin levels in the columella are slightly reduced. This suggests that *PLT* expression in the stem cell niche is not reduced

Figure 5 ADA2b acts in the PLT pathway



(A-C) Root tip of *ada2b-3* (A), *plt1-4 plt2-2* (B) and *ada2b-3 plt1-4 plt2-2* (C), 4 dpg. (D-F) Root tip of *ada2b-3* (D), *shr-2* (E) and *ada2b-3 shr-2* (F), 5 dpg. (G-I) Root tip of *ada2b-3* (G), *scr-4* (H) and *ada2b-3 scr-4* (I), 7 dpg. (J-L) Root tip of *RBR RNAi* (J), *ada2b-3* (K) and *ada2b-3 RBR RNAi* (L), 6 dpg.

due to a change in auxin perception or distribution. Together, these data indicate that ADA2b is required for proper *PLT1* and *PLT2* expression, similar to GCN5. In addition, ADA2b influences meristem size and proliferation of the transit amplifying cells.

ADA2b acts in the PLT pathway

Different combinations with null mutants were made to assess whether ADA2b acts through the PLT pathway or the SHR/SCR/RBR pathway. Shorter roots, smaller meristem size and less cortex cells are found in both *ada2b-3 scr-4* (Figure 5G-I, Supplementary Figure 3E,H) and *ada2b-3 shr-2* double mutant seedlings (Figure 5D-F, Supplementary Figure 3D,G) compared to the single mutants. This suggests that ADA2b acts in a pathway parallel to both SCR and SHR. Although additional columella stem cell layers are transiently found (Figure 5J-L, Supplementary Figure 3K), *RBR* reduction does not increase meristem size, number of cortex cells or root length of

ada2b-3 mutants (Supplementary Figure 3I-J; data not shown). Our data suggest that ADA2b acts independent of the SHR/SCR/RBR pathway, similar to GCN5.

Root size is further reduced in *ada2b-3 plt1-4 plt2-2* triple mutants compared to *plt1-4 plt2-2* double mutants (Supplementary Figure 3C). However, meristem size and number of cortex cells in *ada2b-3 plt1-4 plt2-2* triple mutants and *plt1-4 plt2-2* double mutants is similar (Figure 5A-C, Supplementary Figure 3F). These data indicate that ADA2b acts through PLT1 and PLT2 in the transit amplifying cells and that independent factors are affected in the elongation or differentiation zone, possibly other *PLT* genes (Galinha et al., 2007).

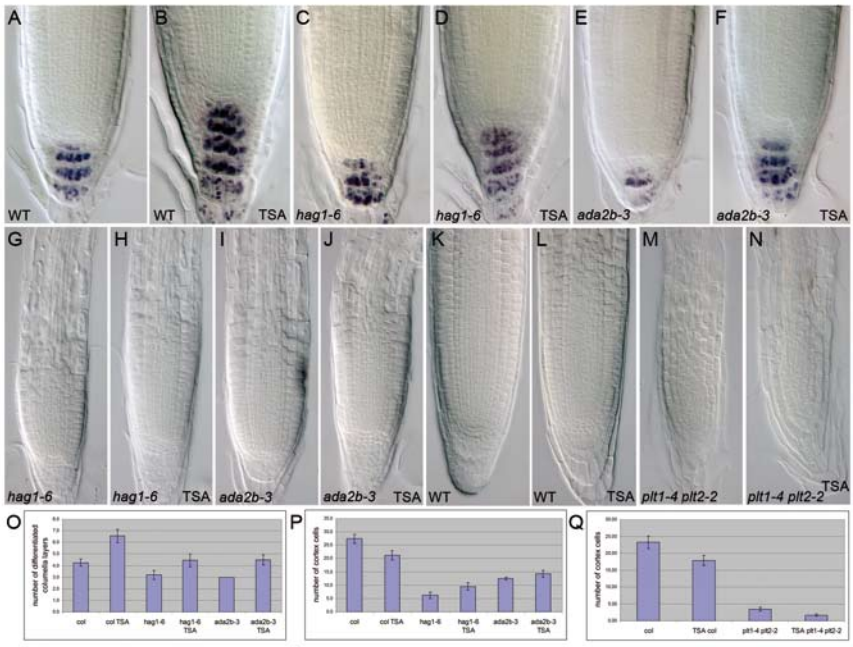
ada2b meristem size is not rescued by overexpression of *PLT2*

We determined whether overexpression of *PLT2* (Galinha et al., 2007) can rescue meristem size of *ada2b-3* mutants. The size of the elongation zone and the number of epidermis cells in the elongation zone of *ada2b-3* mutants are significantly increased due to overexpression of *PLT2* (Figure 3I; *t*-test, $P = 3.2\ 10^{-2}$), similar to *hag1-6* mutants. In contrast, *ada2b-3* mutant seedlings do not show a highly significant increase in meristem size and number of cortex cells (Figure 3F-G,H; *t*-test, $P = 0.04$), when *PLT2* is induced. Furthermore, when *hag1-6* mutant seedlings or their heterozygous siblings are germinated on dexamethasone, seedlings are white and small due to over-expression of *PLT2*, similar to wildtype (Figure 3J). In contrast, *ada2b-3* homozygotes and heterozygotes (as determined by genotyping) are greener and larger, when germinated on dexamethasone (Figure 3J). Thus, the presence of even a single *ada2b-3* allele counteracts the full extent of the phenotype caused by overexpression of *PLT2*. Taken together, our data show that *PLT2* overexpression is unable to rescue *ada2b* meristem size defects, whereas the elongation zone is restored. In addition, it confirms that ADA2b regulates proliferation of the transit amplifying cells.

HDAC inhibitor TSA partially and indirectly rescues meristem defects in *gcn5* and *ada2b* mutants

Gene expression is determined by a balance between HAT and histone deacetylase (HDAC) activity, also in *Arabidopsis* (Benhamed et al., 2006). Global histone acetylation is diminished in *gcn5* (Benhamed et al., 2006; Bertrand et al., 2003) and possibly

Figure 6 HDAC inhibitor TSA indirectly and partially rescues meristem defect of *gcn5* and *ada2b* mutants



(A-F) Differentiated columella layers stained with lugol of Col (A-B), *hag1-6* (C-D), *ada2b-3* (E-F), without (A,C,E) or with 1μM TSA (B,D,F), 7 dpg. (G-J) Meristem of *hag1-6* (G-H) and *ada2b-3* (I-J) without (G,I) or with 1μM TSA (H,J), 7 dpg. (K-N) Meristem of Col (K-L) and *plt1-4 plt2-2* (M-N) without (K,M) or with 1μM TSA (L,N), 4 dpg. (O-P) Quantification number of differentiated columella layers (O) and cortex cells (P) in Col, *hag1-6* and *ada2b-3* without or with 1μM TSA, 7 dpg. (Q) Quantification number of cortex cells in Col and *plt1-4 plt2-2* without or with 1μM TSA, 4 dpg. Error bars indicate 95% confidence interval.

ada2b mutants. To determine whether a compensatory reduction of HDAC activity is able to rescue meristem size in *gcn5* and *ada2b* mutants, the HDAC inhibitor TSA was applied at germination. TSA increases the number of differentiated columella layers in wildtype (Figure 6A-B), *hag1-6* (Figure 6C-D,O; *t*-test, $P = 2.8\ 10^{-2}$) and *ada2b-3* mutants (Figure 6E-F,O; *t*-test, $P = 0.001$). Meristem size and the number of cortex cells are increased in *hag1-6* (Figure 6G-H,P; *t*-test, $P = 4\ 10^{-3}$) and slightly increased in *ada2b-3* mutants (Figure6I-J,P; *t*-test, $P = 0.03$) after TSA application, whereas these are decreased in wildtype (Figure 6P). This suggests that application of TSA and thereby inhibition of HDACs can partially rescue mutation of *GCN5* and *ADA2b*.

However, TSA does not increase the expression of *PLT1* and *PLT2* either in wildtype or in *hag1-6* and *ada2b-3* mutants (data not shown). TSA is unable to rescue meristem size of *plt1-4 plt2-2* mutants, since it induces a slightly smaller meristem (Figure 6M-N,Q). Together, these data suggest that TSA rescues meristem size of *gcn5* and *ada2b* mutants and does not directly affect *PLT* expression.

Discussion

Here, we provide evidence that GCN5 and ADA2b have specific roles in root development despite their pleiotropic phenotypes. We show that GCN5 and ADA2b have overlapping functions: they are positioned in the PLT pathway based on genetic interactions and specifically regulate the level of *PLT1* and *PLT2* expression post-embryonically. This fits with the observation that histone acetylation mediated by GCN5 containing complexes quantitatively facilitates transcription in mammals (reviewed by Baker and Grant, 2007; Nagy and Tora, 2007; Shahbazian and Grunstein, 2007). Recruitment of these complexes to target promoters is mediated by transcription factors, also in *Arabidopsis* (Mao et al., 2006; Stockinger et al., 2001). However, whether GCN5 and ADA2b are present directly at the *PLT1* and *PLT2* promoter is unknown, as well as the responsible transcription factor that recruits GCN5 and ADA2b. The PLT gradient is set up during embryogenesis downstream of auxin accumulation and auxin response factors (Aida et al., 2004; Blilou et al., 2005). In turn, PLT proteins regulate the PIN proteins (putative auxin efflux carriers) and vice versa, thereby fine-tuning the position of the auxin maximum from late embryogenesis onwards (Blilou et al., 2005). The auxin maximum is not affected post-embryonically in both *gcn5* and *ada2b* mutants, suggesting that *PLT1* and *PLT2* levels are regulated by GCN5 and ADA2b independent of auxin.

We show that GCN5 controls stem cell niche maintenance and proliferation of the transit amplifying cells through the PLT pathway. High levels of *PLT2* maintain the stem cells, intermediate levels facilitate the transit amplifying cell divisions, and low levels define the exit to differentiation (Galinha et al., 2007). Overexpression of *PLT2* is able to rescue the stem cell niche defect in *gcn5* mutants, confirming that GCN5 mainly regulates stem cell niche maintenance through its effect on *PLT* levels. *gcn5* mutants differentiate later (14 dpg) than *plt1 plt2* double mutants (7 dpg) (Aida et al., 2004),

probably because residual PLT1 and PLT2 activity is present. Surprisingly, the stem cell niche is maintained in *ada2b* mutants although *PLT1* and *PLT2* expression levels are as severely reduced as in the *gcn5* mutant, causing stem cell loss in this mutant. This suggests that mutation of *ADA2b* counteracts low PLT levels in the stem cells. GCN5 and ADA2b might not always act in the same complex and have similar targets, which is also suggested by microarray experiments (Vlachonasios et al., 2003) and our mutant analysis. Determination whether GCN5 and ADA2b have overlapping target genes in stem cells, should elucidate the stem cell maintenance controversy and the molecular mechanisms involved.

In mammals, pluripotent transcription factors (Oct4 and Sox2) are essential and sufficient to induce the stem cell state and impose the unique chromatin state present in stem cells by regulating chromatin factors (reviewed by Niwa, 2007a; Niwa, 2007b). In analogy, the PLT transcription factors are necessary and sufficient to induce (ectopic) root stem cell niches (Aida et al., 2004; Galinha et al., 2007). Surprisingly, we find that the chromatin factor GCN5 regulates expression of the *PLT* transcription factors, while the opposite is found in mammals (Loh et al., 2007). Possibly, PLT proteins induce *GCN5* expression and GCN5 is necessary to continuously elevate levels of *PLT* to maintain pluripotency in *Arabidopsis* stem cells. In the future, we will need to unravel further the regulatory and molecular mechanisms that are at work between GCN5 and the PLT proteins in stem cells.

We provide more evidence that GCN5 and ADA2b have distinct functions during root development. ADA2b influences meristem size and proliferation of the transit amplifying cells directly. Meristem size of the *ada2b* mutant is not rescued by over-expression of *PLT2*. Instead, transit amplifying cell proliferation induced by *PLT2* over-expression is prevented in *ada2b* mutants, even by the presence of a single mutant allele. This indicates that ADA2b influences the 'read-out' of PLT levels in the transit amplifying cells. Formally, mutation of *ADA2b* could affect another unidentified pathway, which blocks proliferation of transit amplifying cells induced by *PLT2* over-expression. Alternatively and perhaps more likely, mutation of *ADA2b* might influence transit amplifying cell proliferation mediated by intermediate PLT levels by affecting the expression of specific 'intermediate' PLT targets. ADA2b was shown previously to regulate the expression of cell cycle genes (Sieberer et al., 2003). We show that *cycB1;1* expression is completely absent in *ada2b* mutants, confirming that ADA2b

regulates cell cycle genes. Growth and thus cell division is not abolished in *ada2b* mutants, suggesting that cycB1;1 is not essential for cell cycle progression. High expression of *cycB1;1* was shown to enhance shoot and root growth rates (Doerner et al., 1996; Li et al., 2005), indicating that ADA2b regulation of *cycB1;1* and its redundant family members can promote proliferation of transit amplifying cells. PLT targets include cell cycle genes (Marijn Luijten and Renze Heidstra; personal communication), which may mediate the proliferation of transit amplifying cells in response to 'intermediate' PLT levels. ADA2b might regulate the same genes as PLT and in this way influence the 'read-out' of PLT levels and thereby meristem size. In analogy, the stochastic transient rescue of the *gcn5* meristem size by *PLT2* overexpression suggests that GCN5 may also affect expression of 'intermediate' PLT target genes. This scenario predicts that GCN5, ADA2b and PLT protein activity may converge on PLT target genes. In the future, determination of GCN5 and ADA2b target genes in stem cells and transit amplifying cells should elucidate whether the functions of GCN5 and ADA2b are coinciding in these two cellular compartments.

Although mammalian pluripotent transcription factors (Oct4 and Sox2) are sufficient to induce pluripotency, ectopic expression of mammalian oncogenes c-Myc and Klf4 increases the frequency of induced pluripotent stem (iPS) cells (reviewed by Jaenisch and Young, 2008). The transcription factor c-Myc is required for the maintenance of active chromatin in progenitor cells by regulating *GCN5* expression directly (Knoepfler et al., 2006). We show that meristem size of *gcn5* and *ada2b* mutants can be partially rescued through inhibition of HDACs by TSA, which causes global hyperacetylation (Xu et al., 2005). Rescue of transit amplifying cell proliferation seems to be indirect and independent of *PLT* expression. A potential explanation for this independency is that reduced *PLT* levels in *gcn5* and *ada2b* mutants are partially compensated by hyperacetylation of 'intermediate' PLT target genes increasing their expression and thereby proliferation and meristem size. Therefore, *Arabidopsis* GCN5 and its associated factor ADA2b might be required to establish active chromatin in transit amplifying cells to maintain proliferation. In the future, histone modifications on *PLT* genes and PLT target genes need to be investigated in stem cells and transit amplifying cells to determine the exact molecular mechanism of GCN5 and ADA2b action.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotypes Columbia (Col) and Wassilewskija (WS) were used. *hag1-5* (SALK_048427), *hag1-6* (SALK_150784) and *ada2b-3* (SALK_019407) (Col) were obtained from the Signal Insertion Mutant Library (http://signal.salk.edu) and were confirmed by PCR-based genotyping. *ada2b-1* (WS) was kindly provided by Vlachonasios (Vlachonasios et al., 2003). *hag1-6* and *ada2b-3* mutant plants were unable to produce functional flower meristems, therefore they were maintained as a heterozygous population.
Origins and ecotypes of other markers and mutants are as follows: QC25 (WS) (Sabatini et al., 2003); *pSHR::GFP:SHR* (Col) (Nakajima et al., 2001); *pPLT1::PLT1:YFP*, *pPLT2::CFP*, *pPLT2::PLT2:YFP*, *35S::PLT2:GR* (Col) (Galinha et al., 2007); *DR5::GUS* (Col) (Ulmasov et al., 1997); *cyclinB1;1:GUS* (Col) (Colon-Carmona et al., 1999); *scr-4* (WS), *shr-2* (Col) (Fukaki et al., 1998); *RBR RNAi* (Col) (Wildwater et al., 2005); *plt1-4 plt2-2* (WS) (Aida et al., 2004).

Histone deacetylase inhibitor TSA (Trichostatin A) was obtained from Sigma, dissolved in dimethylsulfoxide (stock concentration 5 mM), applied upon germination (1μM TSA) and stored at -20°C until use.
Plants were grown as described before (Sabatini et al., 1999).

Transgenic plants

The *GCN5* translational fusion (*pGCN5::GCN5:GFP*) was constructed by PCR-amplifying 3.3 kb of the promoter using primers
(5'-GGGGACAACTTTGTATAGAAAAGTTGTTccgttcaatttaaagaaatccaacaa-3') and
(5'-GGGGACTGCTTTTTTGTACAAACTTGcgaagcagtatagtgaaggtgattga-3')
and the *GCN5* genomic coding sequence using primers
(5'-ggggacaagtttgtacaaaaaagcaggctATGGACTCTCACTCTTCCCACCTC-3') and
(5'-ggggaccactttgtacaagaaagctgggttTTGAGATTTAGCACCAGATTGGAGA-3')
from Col genomic DNA. These fragments were placed in a pGreenII vector (Hellens et al., 2000) containing norflurazon resistance (Heidstra et al., 2004) together with a GFP coding sequence (fused to the 3'-end).

In the same way the *ADA2b* translational fusion (*pADA2b::ADA2b:GFP*) was constructed by PCR-amplifying 1.8 kb of the promoter using primers (5'-GGGGACAACTTTGTATAGAAAAGTTGttctcctgaaacgttcattgacat-3') and (5'-GGGGACTGCTTTTTTGTACAAACTTGtgatgcccaagtagaaaattggatt-3') and the *ADA2b* genomic coding sequence using primers (5'-ggggacaagtttgtacaaaaaagcaggctATGGGTCGCTCTCGAGGGAAC-3') and (5'-ggggaccactttgtacaagaaagctgggttAAGTTGAGCAATACCCTTCTTCACA-3') from Col genomic DNA.

For the construction of *pSCR::GFP*, a 2.4 kb HindIII fragment of the *SCR* promoter was generated by PCR using primers pSCRF (5'-TCTCTATGAAAAGTGGAAATTTACCT-GGAA-3') and pSCRR (5'-GGAGATTGAAGGGTTGTTGGTCGTG-3') and cloned into *pGII226-GFP$_{ER}$T*.

To construct the *pPLT2::PLT2:GFP* translational fusion, a 5876 bp fragment of the *PLT2* promoter, the *PLT2* genomic coding sequence and GFP were cloned into *pGII0227*. Transgenic plants were generated using the floral dip method (Clough and Bent, 1998).

Microscopy and in situ hybridization

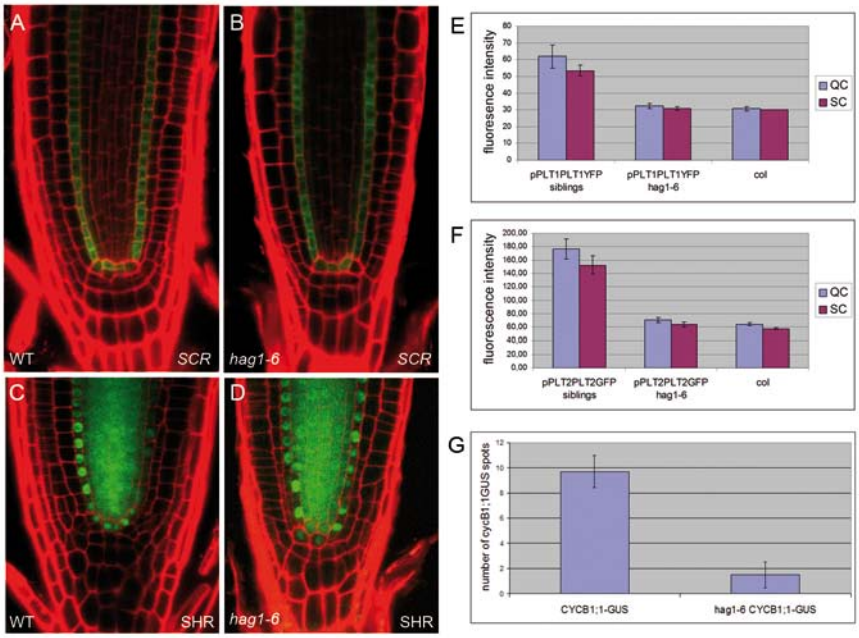
Light microscopy and fluorescence microscopy was performed as described (Willemsen et al., 1998). For confocal microscopy, dissected embryos were mounted in 7% glucose. Starch granules and β-glucuronidase activity were visualised as before (Willemsen et al., 1998). Root length, meristem size and differentiated epidermis cell size was determined as described (Willemsen et al., 1998). Fluorescence levels were quantified as before (Galinha et al., 2007). Whole-mount in situ hybridisation was performed manually (Hejatko et al., 2006) using *PLT1* probe as described (Aida et al., 2004).

Acknowledgements

We are grateful to K. Vlachonasios for *ada2b-1* seeds. We thank R. Leito and F. Kindt for technical assistance.

Supplementary Figure 1

GCN5 does not affect *SCR* expression and SHR localization, but regulates *PLT 1, PLT2* and *cycB1;1* expression



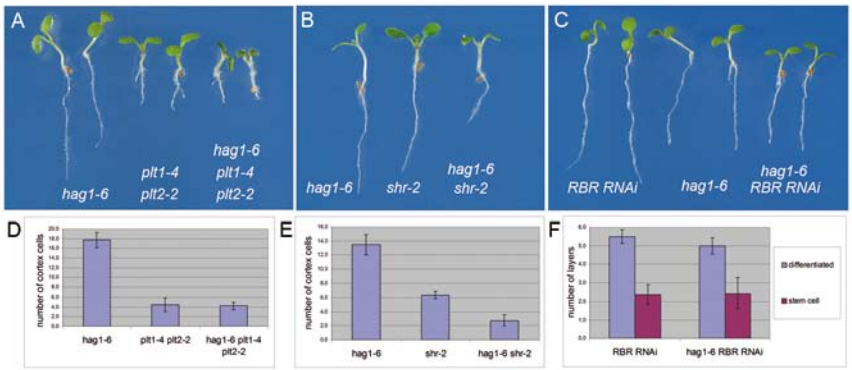
(A-B) Expression of *pSCR::GFP* in wildtype (A) and *hag1-6* (B) 7dpg. (C-D) Expression of *pSHR::GFP:SHR* in wildtype (C) and *hag1-6* (D) 7 dpg. (E-F) Quantification of *pPLT1::PLT1:YFP* (E) and *pPLT2::PLT2:GFP* (F) in *hag1-6* mutant seedlings 4 dpg compared to their siblings, SC indicates columella stem cell. (G) Quantification number of *cycB1;1:GUS* spots in wildtype and *hag1-6*. Error bars indicate 95% confidence interval.

Supplementary Table 1 GCN5 is required for stem cell niche maintenance

	percentage (%)
QC +,+; SC +	20,0
QC +,+; SC -	3,3
QC +,-; SC +	26,7
QC +,-; SC -	3,3
QC -[a],+; SC +	20,0
QC -[a],+; SC -	10,0
QC -,+; SC +	10,0
QC -,+; SC -	3,3
QC -[b],+; SC +	3,3

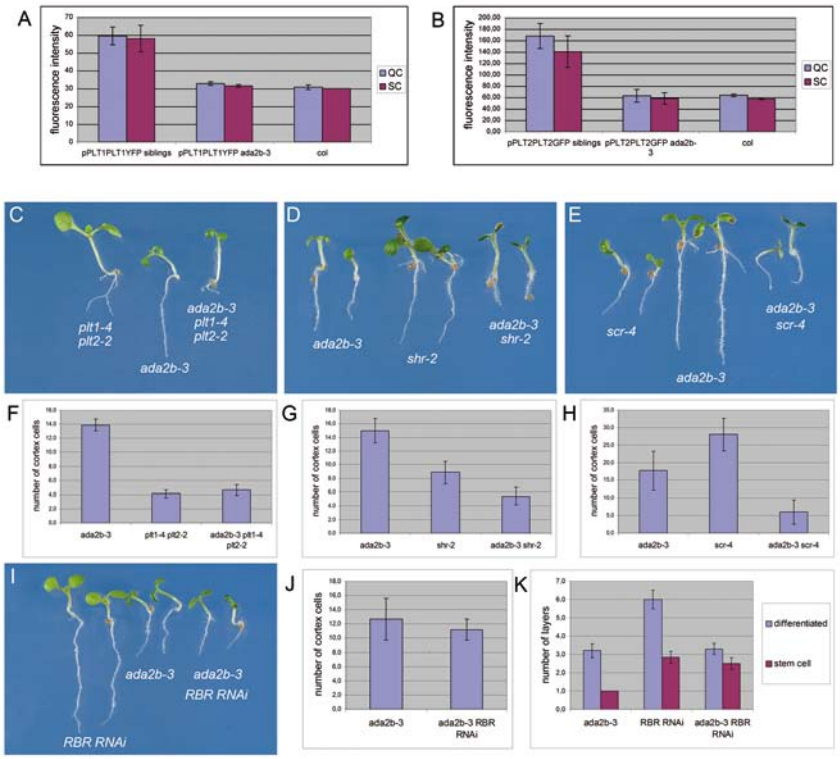
Quantification of stem cell niche defect in *hag1-6* seedlings 7 dpg (n=30).
"+" or "–" indicates whether QC and stem cell (SC) is specified or not, respectively. Specification of QC is determined by two criteria: first, QC25 expression on (+) or off (-); second: starch absent (+) or present (-). Specification of stem cell (SC) is classified by absence (+) or presence of starch (-).[a] some QC cells lack QC25 expression, [b] divided QC.

Supplementary Figure 2 GCN5 acts in the PLT pathway



(A) Root length of *hag1-6*, *plt1-4 plt2-2* and *hag1-6 plt1-4 plt2-2*, 7 dpg. (B) Root length of *hag1-6*, *shr-2* and *hag1-6 shr-2*, 7 dpg. (C) Root length of *RBR RNAi* , *hag1-6 and hag1-6 RBR RNAi*, 7 dpg. (D-E) Quantification number of cortex cells of *hag1-6 plt1-4 plt2-2*, 4 dpg (D), *hag1-6 shr-2*, 7 dpg (E) and single/double mutants. (F) Quantification number of differentiated columella layers and stem cell layers in *RBR RNAi* and *hag1-6 RBR RNAi*, 7 dpg. Error bars indicate 95% confidence interval.

Supplementary Figure 3 ADA2b regulates *PLT1* and *PLT2* expression, and acts in the PLT pathway



(A-B) Quantification of *pPLT1::PLT1:YFP* (A) and *pPLT2::PLT2:GFP* (B) in *ada2b-3* mutant seedlings compared to their siblings, 4 dpg, SC indicates columella stem cell. (C-E) Root length of *ada2b-3 plt1-4 plt2-2* (C), *hag1-6 shr-2* (D), *hag1-6 scr-4* (E) and singles/doubles, 7 dpg. (F-H) Quantification number of cortex cells of *ada2b-3 plt1-4 plt2-2,* 4 dpg (F), *ada2b-3 shr-2*, 5 dpg (G), *ada2b-3 scr-4*, 7 dpg (H) and single mutants. (I) Root length of *ada2b-3 RBR RNAi* and singles, 7 dpg. (J) Quantification number of cortex cells of *ada2b-3 RBR RNAi*, 6 dpg. (K) Quantification number of differentiated columella layers and stem cell layers in *ada2b-3 RBR RNAi* and single mutants, 7 dpg. Error bars indicate 95% confidence interval.

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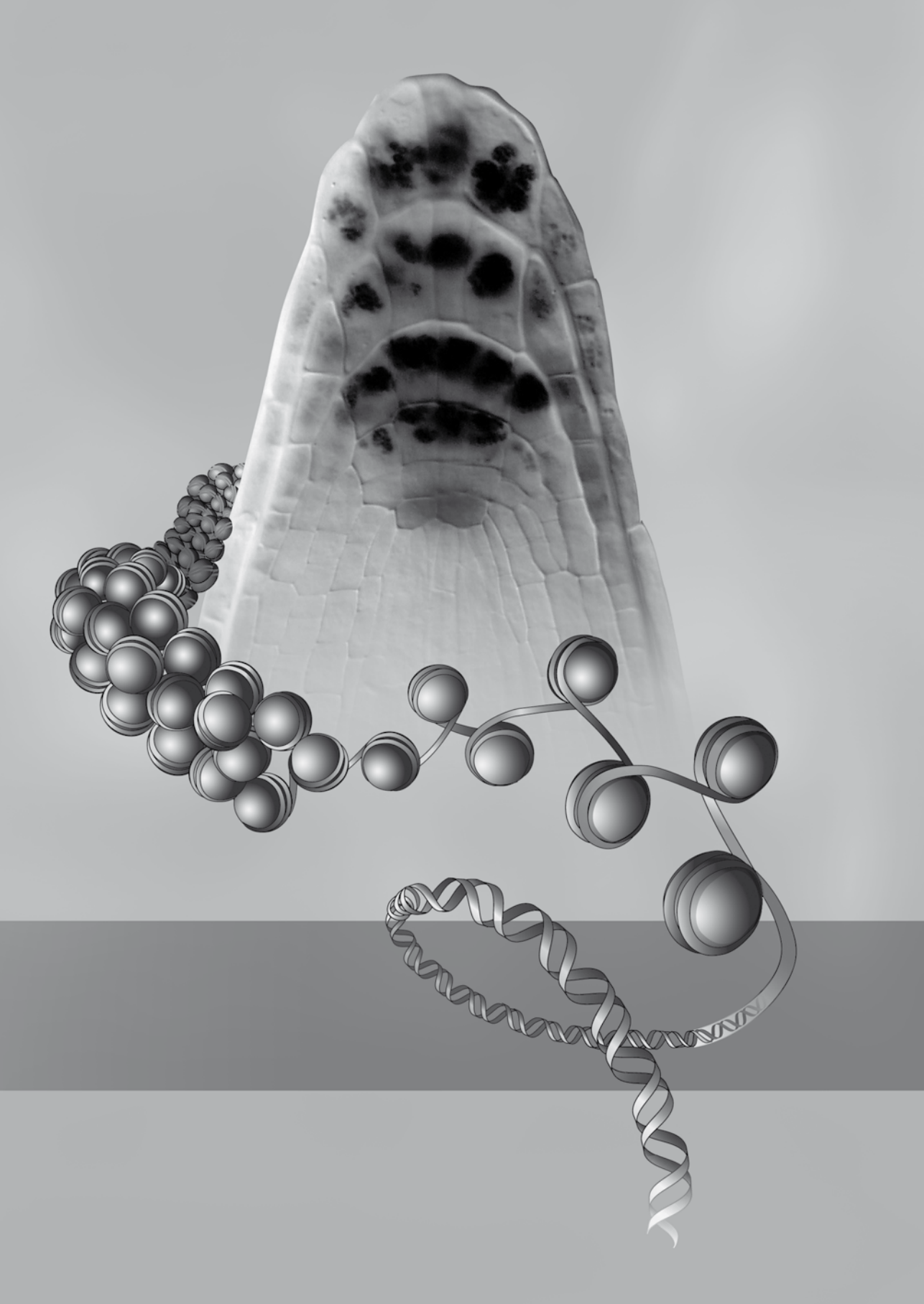

CHAPTER 3

The *Arabidopsis* CAF-1 complex and RBR synergistically repress stem cell proliferation and promote differentiation in the *Arabidopsis* root meristem

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Summary

The *Arabidopsis* CAF-1 complex facilitates chromatin assembly after replication similar to its animal and yeast counterparts. In addition, the complex is involved in developmental processes and regulates expression of a small subset of genes. Here, we show that the *Arabidopsis* CAF-1 complex and RETINOBLASTOMA RELATED (RBR) repress synergistically stem cell proliferation in the root through regulation of stem cell niche transcription factors and promote timely differentiation. Our data indicate that the CAF-1 complex and RBR act in the same pathway and have partially overlapping targets. Concerted regulation of these targets might be facilitated by a direct interaction between the largest subunit of the CAF-1 complex and RBR. Acute reduction of the CAF-1 complex and RBR, and treatments with the histone deacetylase inhibitor TSA show that stem cell loss and proliferation are probably caused by an accumulation of epigenetic defects. Together, our data highlight an important role for epigenetic mechanisms in plant stem cell control and proliferation.

Introduction

Chromatin needs to be reconstituted during replication to ensure proper inheritance of epigenetic states. The main factor responsible for replication-coupled histone H3 and H4 deposition onto newly replicated DNA is chromatin assembly factor 1 (CAF-1). The CAF-1 complex consists of p150, p60 and p48 subunits in mammals, and Cac1, Cac2 and Cac3 subunits in yeast (reviewed by Ridgway and Almouzni, 2000). The largest subunit is recruited to the replication fork through interaction with replication factor PCNA. In yeast, the CAF-1 complex is involved in silencing at telomeres and the mating-type locus, while cell cycle arrest, S-phase checkpoint activation and cell death take place when the mammalian CAF-1 complex is impaired (reviewed by Ramirez-Parra and Gutierrez, 2007b; Ridgway and Almouzni, 2000).

In *Arabidopsis*, the three CAF-1 subunits are encoded by *FASCIATA1* (*FAS1*), *FAS2* and *MSI1*, respectively (Kaya et al., 2001; Ramirez-Parra and Gutierrez, 2007b). MSI1 is a histone chaperone found in many other chromatin complexes, like histone deacetylase complexes and Polycomb complexes (reviewed by Hennig et al., 2005). Mutation of *FAS1* and *FAS2* results in an increased frequency of homologous recombination and double-strand breaks (DSBs) (Endo et al., 2006; Kirik et al., 2006), indicating that the *Arabidopsis* CAF-1 complex is required for genome stability. In *fas* mutants, heterochromatin compaction is reduced, although chromatin modifications are not globally affected (Kirik et al., 2006; Schonrock et al., 2006). In addition, the CAF-1 complex is involved in many developmental processes and ensures stable maintenance of epigenetic states of a small subset of genes (Costa and Shaw, 2006; Exner et al., 2006; Kaya et al., 2001; Ramirez-Parra and Gutierrez, 2007a; Schonrock et al., 2006). Transcription of 2.1% and 0.9% of all genes is affected in *fas1* and *fas2* mutants, respectively. These genes are enriched for expression in the late S-phase and include genes involved in DNA repair (Schonrock et al., 2006). The epigenetic state of only a small fraction of genes is changed in *fas* mutants, whereas their neighbouring genes are not affected (Ramirez-Parra and Gutierrez, 2007a). *FAS1* is transcribed specifically in the S-phase (Kaya et al., 2001) and is regulated by E2F transcription factors (Ramirez-Parra and Gutierrez, 2007a). Furthermore, it is thought that defects in the CAF-1 complex activate the DNA damage checkpoint and induce endoreduplication to overcome irreversible cell cycle arrest (Endo et al., 2006; Exner et al., 2006; Kirik et al., 2006; Ramirez-Parra and Gutierrez, 2007a).

Mutation of either *fas1* or *fas2* causes defects in root size and stem cell niche maintenance (Kaya et al., 2001). The root stem cell niche is defined by the PLETHORA (PLT) pathway (Aida et al., 2004; Blilou et al., 2005) and the SHORTROOT (SHR)/ SCARECROW (SCR)/ RETINOBLASTOMA RELATED (RBR) pathway (Helariutta et al., 2000; Nakajima et al., 2001; Sabatini et al., 2003; Wildwater et al., 2005). RBR acts genetically downstream of the SHR target SCR and regulates the size of the stem cell pool (Wildwater et al., 2005).

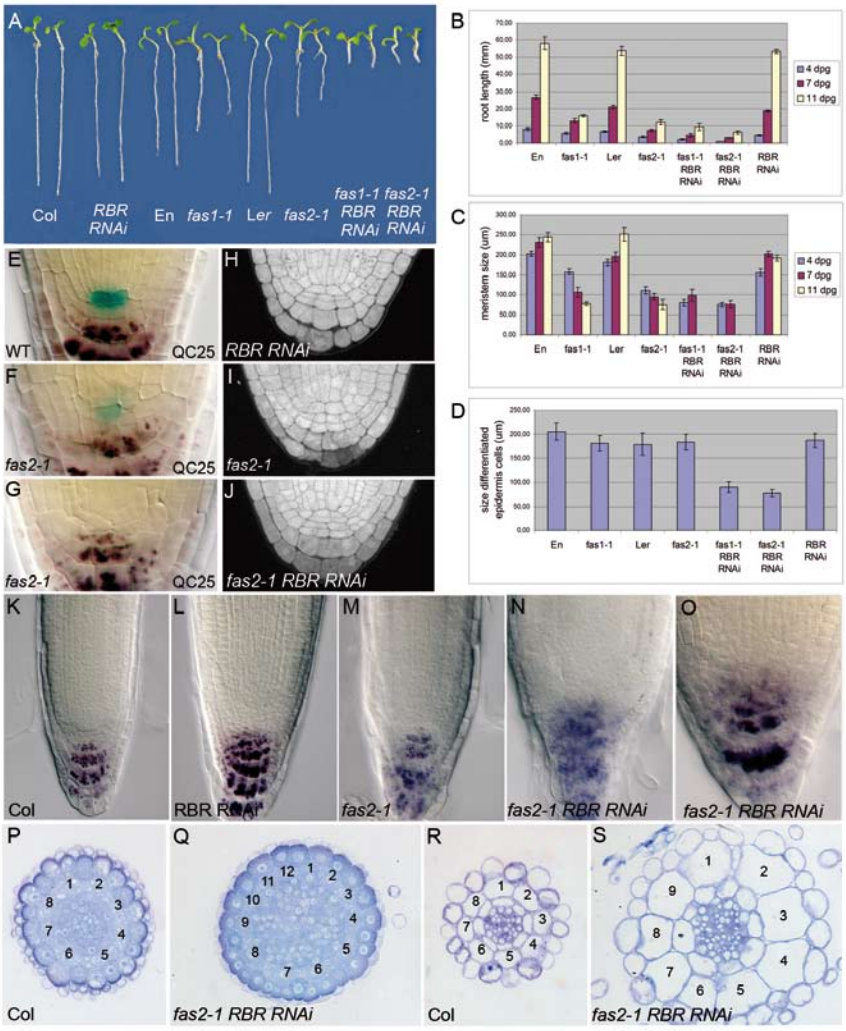
Here, we investigated in detail the *fas* stem cell niche defect and whether it is caused by misregulation of the transcription factors known to be required for root stem cell maintenance. We show that the stem cell niche defect of *fas* mutants is independent of *PLT1, SCR* and *SHR* expression, despite the previously reported aberrant *SCR* expression in *fas* mutants (Kaya et al., 2001). We find that *SCR* expression is unaltered in the primary root around the time of stem cell loss, suggesting that loss and ectopic *SCR* expression is a late effect and is not related to the *fas* stem cell niche defect. Furthermore, we provide evidence that the CAF-1 complex acts in the SHR/SCR/RBR pathway and independent of the PLT pathway. The CAF-1 complex and RBR influence synergistically the proliferation of stem cells through regulation of stem cell transcription factors (including PLT and SCR) and promote correct differentiation. This indicates that the CAF-1 complex and RBR have partially overlapping targets and concerted regulation of these targets might be mediated by the direct interaction between FAS1 and RBR. Acute reduction of the CAF-1 complex or RBR, as well as induced DNA damage do not lead to stem cell loss or overproliferation. However, RBR and a HDAC activity synergistically repress proliferation similar to the CAF-1 complex and RBR, suggesting that stem cell proliferation is caused by accumulation of epigenetic defects.

Results

The CAF-1 complex maintains the stem cell niche, but not through regulation of *PLT, SHR* and *SCR* expression

Previously, Kaya et al. (2001) reported a defect in stem cell niche maintenance and *SCR* expression in the *fas* mutants. To investigate in detail the role of the CAF-1 complex in root development, we analysed *fas1-1* (En), *fas1-1* three times backcrossed

Figure 1 The CAF-1 complex is involved in stem cell maintenance and synergistically represses stem cell proliferation with RBR



(A) Phenotype of wildtype, *fas* mutants and *fas RBR RNAi* mutants, 7 dpg. (B-D) Root length (B) meristem size (C) and size of differentiated epidermis cells 7 dpg (D) of *fas* and *fas RBR RNAi* seedlings compared to wildtype, error bars indicate 95% confidence interval. (E-G) QC25 expression and starch granules in wildtype (E) and *fas2-1* mutant (F-G), 4 dpg. (H-J) Analine blue staining of mature *RBR RNAi* (H), *fas2-1* (I) and *fas2-1 RBR RNAi* (J) embryos. (K-M) Root tip of Col (K), *RBR RNAi* (L), *fas2-1* (M), *fas2-1 RBR RNAi* (N), 5 dpg. (O) Root tip of *fas2-1 RBR RNAi*, 3 dpg. (P-S) Cross section just above the stem cells (P-Q) or in the elongation zone (R-S) of Col (P,R) and *fas2-1 RBR RNAi* (Q,S) seedlings, 3 dpg.

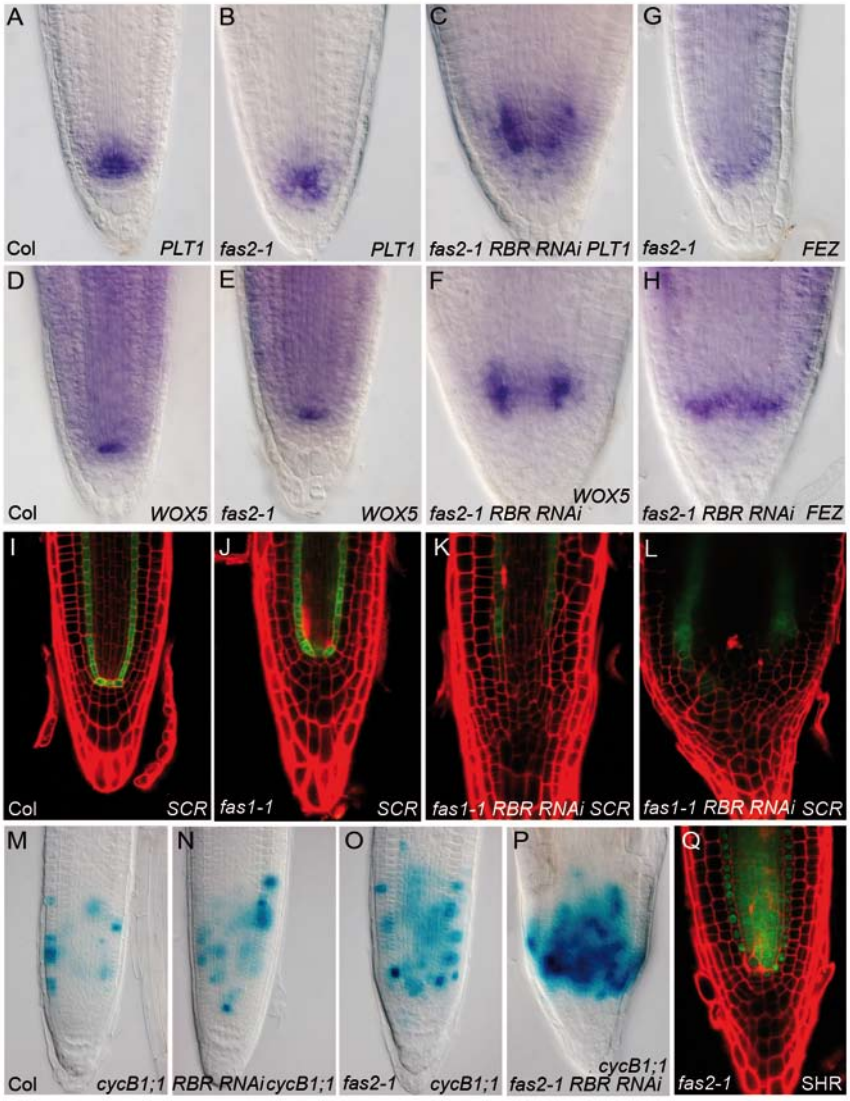
to Col, *fas2-1* (L*er*) and *fas2-4* (Col, SALK_033228, T-DNA insertion in the 6th exon). Both *fas1* and *fas2* mutants show shorter root length as reported previously (Figure 1A-B; data not shown) (Kaya et al., 2001; Leyser and Furner, 1992; Ramirez-Parra and Gutierrez, 2007a) and smaller meristem size (Figure 1C; data not shown). To examine the stem cell niche defect in the *fas* mutants, a marker specific for the QC (QC25) was analysed in combination with a columella differentiation marker (starch granules). In wildtype, a layer of columella stem cells is present between the QC and differentiated columella cells (Figure 1E). In contrast, both *fas1-1* and *fas2-1* mutants show starch granules next to the QC (84%, n=25, *fas2-1*) (Figure 1F; data not shown) or QC25 is even absent (28%, n=25, *fas2-1*) (Figure 1G; data not shown) 4 dpg, indicating a stem cell niche defect and columella stem cell loss. In addition, extra divisions in the endodermis can be occasionally found (data not shown).

To determine whether the stem cell niche defect in the *fas* mutants is caused by misregulation of the stem cell niche-defining transcription factors PLT, SHR and SCR, we analysed their expression. In situ hybridization with a *PLT1* probe did not reveal changes in *PLT1* expression in both *fas1-1* and *fas2-1* mutants compared to wildtype (Figure 2A-B; Supplementary Figure 2A-B). PLT proteins regulate the PIN proteins (putative auxin efflux carriers) and vice versa, thereby fine-tuning the position of the auxin maximum (Aida et al., 2004; Blilou et al., 2005). The auxin maximum as determined by *DR5::GUS* (Ulmasov et al., 1997) is not affected in the *fas* background (Supplementary Figure 2N-P). Together, our data indicate that the CAF-1 complex acts independent of the PLT pathway.

In addition, neither expression of *SCR* (Figure 2J; Supplementary Figure 2L) nor localization of SHR (Figure 2Q; data not shown) is altered in *fas1-1* and *fas2-1* mutant primary roots 7 dpg compared to wildtype. The *fas* stem cell niche defect is found at 4 dpg and we detect wildtype expression of *SCR* at 7 dpg in both *fas1-1* and *fas1-2* mutant primary roots. Therefore, aberrant *SCR* expression as found by Kaya et al. (2001) appears to be a late effect, that is unrelated to the early stem cell niche defect. Thus, the CAF-1 complex does not potentiate stem cell niche maintenance through regulation of *SCR* and *SHR*.

To investigate cell cycle progression, the expression of *cyclinB1;1:GUS* (marks the G2/M phase of the cell cycle) was examined in the *fas* background. Expression is

Figure 2 The CAF-1 complex and RBR synergistically regulate expression of stem cell niche transcription factors



(A-H) In situ hybridisation using *PLT1* probe (A-C), *WOX5* probe (D-F) and *FEZ* probe (G-H) in Col (A,D), *fas2-1* (B,E,G) and *fas2-1 RBR RNAi* (C,F,H), 2 dpg. (I-L) Expression of *pSCR::GFP* in Col (I) 4 dpg, *fas1-1* (J) 7 dpg, *fas1-1 RBR RNAi* (K-L) 4 dpg. (M-P) Expression of *cycB1;1:GUS* in Col (M), *RBR RNAi* (N), *fas2-1* (O), *fas2-1 RBR RNAi* (P), 4 dpg. (Q) Expression of *pSHR::SHR:GFP* in *fas2-1*, 8 dpg.

frequently found in the QC of *fas* mutants (Figure 2M-O; Supplementary Figure 2I-J), which is also observed in about 30% of *RBR RNAi* seedlings (Wildwater et al., 2005). In addition, *cycB1;1*:*GUS* is present in more cells of *fas* mutants, as reported previously in roots as well as other tissues (Endo et al., 2006; Ramirez-Parra and Gutierrez, 2007a; Schonrock et al., 2006). Elevation of *cycB1;1* expression is also reported to occur due to DNA damage (Ramirez-Parra and Gutierrez, 2007a; Schonrock et al., 2006).

The CAF-1 complex and RBR synergistically repress proliferation of stem cells in the proximal meristem

Reduction of *RBR* results in the gradual acquisition of additional stem cell layers in the root (Figure 1L) (Wildwater et al., 2005). RB interacts with many chromatin factors, including histone chaperone MSI1, which is also found in the CAF-1 complex in both animals and plants (reviewed by Hennig et al., 2005). Therefore, we investigated the genetic interaction between *fas* mutants and *RBR RNAi*. *fas1-1 RBR RNAi*, *fas2-1 RBR RNAi* and *fas2-4 RBR RNAi* show a severely distorted organisation of the stem cell niche area (Figure 1K-N; Supplementary Figure 1A-B; data not shown), which can be already observed at 3 dpg (Figure 1O; Supplementary Figure 1C). Distally, the root cap has more differentiated columella layers in *fas RBR RNAi* seedlings compared to the singles (Figure 1K-N; Supplementary Figure 1A-B). At later stages (7 dpg), novel phenotypes such as splitting of the distal root tip in two physically separated root tips can be occasionally observed, especially in *fas2-1 RBR RNAi* seedlings (Supplementary Figure 1D). Analysis of mature embryos reveals that a higher percentage of QC divisions take place in *fas RBR RNAi* embryos (Figure 1H-J; Supplementary Figure 1M-P; Supplementary Table 1), indicating an early defect in cell division regulation. Root size (Figure 1A-B) and meristem size (Figure 1C) is smaller in *fas RBR RNAi* seedlings, and they differentiate at 11 dpg. The cell size of differentiated epidermis cells is smaller in *fas RBR RNAi* seedlings 7 dpg (Figure 1D), contributing to the shorter root size. In addition, the root tip is broader and contains more cell layers radially (Figure 1K-N; Supplementary Figure 1A-B). Cross sections reveal a substantial increase in the number of cortex cell files just above the stem cell niche in *fas RBR RNAi* seedlings 3 dpg compared to the singles (Figure 1P-Q; Supplementary Figure 1E-H), indicating that the number of cortex stem cells is increased. In 1 out of 4 *RBR RNAi*, *fas1-1* and *fas2-1* roots, 9 cortex cell files were present instead of 8 files in

wildtype (data not shown). The number of cell files decreases near the elongation zone in *fas RBR RNAi* seedlings (Figure 1R-S; Supplementary Figure 1I-L) confirming that the increased number of cell files derive from the stem cell niche area and gradually increase after RBR inactivation. Together, these data show that RBR and the CAF-1 complex synergistically repress proliferation of stem cells.

The CAF-1 complex and RBR synergistically regulate expression of stem cell niche transcription factors and promote differentiation

To examine the distorted stem cell niche area in *fas RBR RNAi* seedlings, expression of several transcription factors was analysed. The homeobox gene *WOX5* is specifically expressed in the QC (Figure 2D) and is necessary for stem cell niche maintenance in the SHR/SCR pathway (Sarkar et al., 2007). In situ hybridization with a *WOX5* probe and a *PLT1* probe revealed that expression of both transcription factors expanded laterally and disappeared from the designated QC area in *fas1-1 RBR RNAi* and *fas2-1 RBR RNAi* seedlings 2 dpg (Figure 2C,F; Supplementary Figure 2C,F), although expression of both transcription factors was unaltered in the single mutants (Figure 2A-B,D-E; Supplementary Figure 2A-B,D-E). In addition, expression of *FEZ*, a NAC transcription factor involved in columella stem cell maintenance (Willemsen et al., submitted), is broader in *fas RBR RNAi* seedlings 2 dpg (Figure 2G-H; Supplementary Figure 2G-H), as determined by in situ hybridisation. Expression of *SCR* disappears from the stem cell niche area in *fas RBR RNAi* at 4 dpg (Figure 2K-L; Supplementary Figure 2M), but is present at 2 dpg (data not shown) and is not affected either in *fas* mutants or in *RBR RNAi* seedlings (Figure 2J; data not shown). The auxin maximum as detected by *DR5::GUS* (Ulmasov et al., 1997) shifts proximally in the *fas RBR RNAi* root tip (Supplementary Figure 2Q) and occasionally forms two auxin peaks when the root tip is broad (Supplementary Figure 2R). The level of *cycB1;1*:*GUS* expression is increased and the number of cells with *cycB1;1*:*GUS* expression is higher in *fas RBR RNAi* seedlings 4 dpg (Figure 2P; Supplementary Figure 2K) compared to the singles.

Together, these data indicate that *fas RBR RNAi* seedlings reposition their stem cell niche similar to what occurs after QC ablation (Xu et al., 2006). However, QC respecification is incomplete and stem cell overproliferation takes place. We conclude that the CAF-1 complex and RBR synergistically confine the expression of stem cell

niche transcription factors and promote correct differentiation, indicating that the CAF-1 complex and RBR have partially overlapping targets.

FAS1 and RBR proteins can interact directly

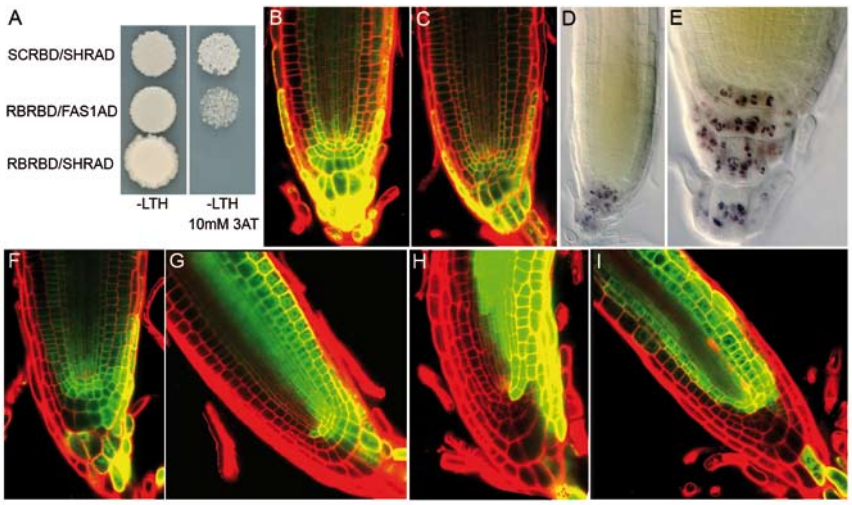
Histone chaperone MSI1 is known to be part of many chromatin complexes, including the CAF-1 complex, and it also interacts with RB in both animals and plants (reviewed by Hennig et al., 2005). However, reduction of MSI1 levels by 30-50% using msi1-antisense plants (Exner et al., 2006) does not lead to enhancement of the phenotype of *fas*, *RBR RNAi* or *fas RBR RNAi* seedlings (data not shown). This indicates that it is unlikely that the CAF-1 complex and RBR regulate overlapping target genes through a mutual interaction with MSI1.

Interestingly, FAS1 contains a LxCxE motif, which is an essential RB interaction motif in several organisms (reviewed by Dick, 2007; Giacinti and Giordano, 2006). In order to investigate whether FAS1 interacts with RBR, a yeast two-hybrid assay was performed. SHR and RBR were included as negative controls, while the interaction between SHR and SCR was used as a positive control (Figure 3A) (Cui et al., 2007; Welch et al., 2007). FAS1 and RBR showed similar growth as the positive control (Figure 3A), indicating that FAS1 and RBR proteins interact *in vitro*. These data suggest that concerted regulation of overlapping targets might be mediated by a direct interaction between the CAF-1 complex and RBR.

Acute deletion of *FAS2* or *RBR* does not result in rapid stem cell niche defects or overproliferation

To investigate the primary defects caused by the loss of the CAF-1 complex, we examined *FAS2* deletion clones. The *fas2-1* mutant was complemented with a vector containing the *FAS2* gene under its own promoter and inserted between *lox* sites as described for SCR and RBR clones (Heidstra et al., 2004; Wildwater et al., 2005). Introduction (by crossing) of a vector containing CRE recombinase driven by a heat-shock promoter in the *fas2-1* background, enabled us to excise the *FAS2* gene locally using heat shock (HS). The resulting *fas2*$^{-/-}$ cells in which recombination has taken place are marked with GFP. When a long HS of 1 hour was applied at 3 dpg seedlings to induce clones in the whole root, meristem and root size decreased after

Figure 3 FAS1 and RBR interact directly, acute deletion of *FAS2* and *RBR* does not cause stem cell loss and overproliferation



(A) Yeast two-hybrid assay. Growth on selective medium -LTH supplemented with 10 mM 3AT shows an interaction *in vitro* between FAS1 and RBR. SHR/ SCR and RBR/SHR are positive and negative controls, respectively. (B-E) *FAS2* deletion clones in the whole root 9 dpg, 7 days after 1 hour heat shock. No phenotype (B) root cap bending (C), smaller meristem (D), stem cells are maintained (E). (F-G) *FAS2* deletion clones in part of the root 9 dpg, 7 days after 30 min heat shock, show root cap bending towards the clone. (H-I) *RBR* deletion clones in *fas2-1* background 9 dpg, 6 days after 20 min heat shock, show occasional root cap bending towards the clone (H).

5 days (Figure 3D; data not shown), similar to *fas2-1* mutants. In addition, 31% of *fas2*$^{-/-}$ clones induced in the whole root display bending of the root cap (Figure 3B-C; n=16). However, stem cell loss was never observed (Figure 3E), suggesting that the CAF-1 complex does not have an acute effect on stem cell niche maintenance. To investigate the cell-autonomous effect of *FAS2* deletion, a shorter HS (30 min) was applied to 3 dpg plants. After 5 days bending of the root cap can be observed towards the cells with excised *FAS2* (Figure 3F-G; n=8), which was also found after acute reduction of *RBR* (Wildwater et al., 2005). Again, no evidence for cell-autonomous QC misspecification and stem cell loss can be found (data not shown), indicating that either DNA stress or accumulation of epigenetic defects causes the stem cell defect in *fas* mutants. On the other hand, the short term effect of acute reduction of *FAS2* is remarkably similar to acute *RBR* deletion.

The combination of *fas* and *RBR RNAi* causes overproliferation of stem cells. To test the effect of acute *RBR* loss in *fas* mutants, we crossed *RBR* deletion clones into the *fas2-1* background (Wildwater et al., 2005). Six days after application of 20 minutes HS 3 dpg, root cap bending occurs (Figure 3H-I; n=3), whereas exaggerated stem cell overproliferation as found in *fas RBR RNAi* seedlings was never found. Our data indicate that stem cell proliferation in *fas RBR RNAi* seedlings is a slow effect either induced by DNA stress or by an accumulation of epigenetic defects.

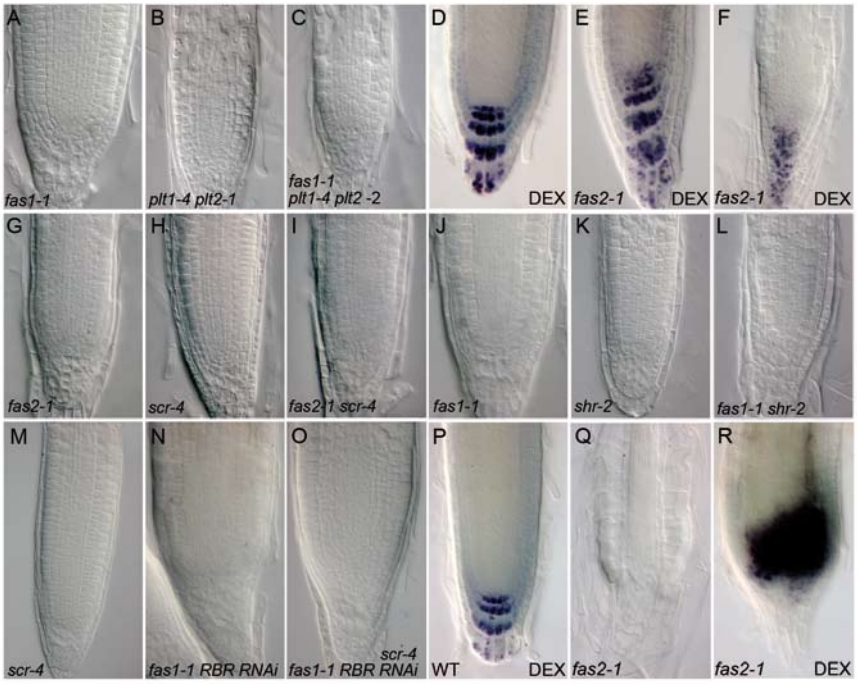
DNA damage and inhibition of DNA replication do not cause stem cell overproliferation in *RBR RNAi* background

Previously, it was shown that DNA damage partially phenocopies the *fas* phenotype in terms of ploidy levels, upregulation of a similar set of genes and a change in their epigenetic state (Ramirez-Parra and Gutierrez, 2007a; Schonrock et al., 2006). To investigate whether the overproliferation phenotype of *fas RBR RNAi* seedlings is a DNA stress effect, several DNA damage agents and inhibitors of DNA replication were used. Aphidicolin is a DNA polymerase inhibitor, hydroxyurea (HU) reduces the dNTP pool and causes DNA replication stress, zeocin induces DSBs, and MMS adds methyl groups to DNA causing DNA damage. All these agents cause differentiation of wildtype and *RBR RNAi* roots (Supplementary Figure 3A-J). However, overproliferation as found in *fas RBR RNAi* seedlings was never found, suggesting that this is not caused by DNA stress. Interestingly, application of aphidicolin to wildtype roots phenocopies aspects of the *fas* mutant phenotype, including smaller meristem, disorganised root cap and stem cell niche (Supplementary Figure 3E; data not shown). Especially in the En background stem cells are lost frequently (Supplementary Figure 3O), suggesting that inhibition of DNA replication might mimic the *fas* mutant phenotype. However, aphidicolin does not cause overproliferation in *RBR RNAi* seedlings (Supplementary Figure 3P).

HDAC activity and RBR synergistically repress proliferation

RB is known to interact with histone deacetylases (HDACs) in animals (Frolov and Dyson, 2004; Giacinti and Giordano, 2006) and also in maize (Rossi et al., 2003). In addition, histone chaperone MSI1 has been found to bind RB, HDACs and the CAF-1 complex in both animals and plants (reviewed by Hennig et al., 2005). To investigate

Figure 4 The CAF-1 complex and RBR act in the SHR/SCR pathway and independent of the PLT pathway



(A-C) Root tip of *fas1-1* (A), *plt1-4 plt2-2* (B) and *fas1-1 plt1-4 plt2-2* (C), 4 dpg. (D-F) Root tip of *35S::PLT2:GR* (D) and *fas2-1 35S::PLT2:GR* (E-F) seedlings 10 dpg, 7 days after application of dexamethasone (DEX). Note stem cell loss (E) and decreased meristem size (F) in *fas2-1 35S::PLT2:GR*. (G-I) Root tip of *fas2-1* (G), *scr-4* (H) and *fas2-1 scr-4* (I), 4 dpg. (J-L) Root tip of *fas1-1* (J), *shr-2* (K) and *fas1-1 shr-2* (L), 4 dpg. (M-O) Root tip of *scr-4* (M) *fas1-1 RBR RNAi* (N) and *fas1-1 RBR RNAi scr-4* (O), 3 dpg. (P-R) Root tip of *35S::PLT2:GR* (P) and *fas2-1 RBR RNAi 35S::PLT2:GR* (Q-R), without (Q) or 7 days after application of dexamethasone (DEX) (P,R), 11 dpg.

whether HDAC activity contributes to stem cell phenotypes, we tested the effect of the HDAC inhibitor TSA on *RBR RNAi* seedlings. In wildtype seedlings, TSA treatment results in additional layers of differentiated columella layers, extra divisions in the endodermis and a moderate reduction of meristem size (Supplementary Figure 3K-L and data not shown). However, enhanced proliferation can be observed in the proximal meristem in *RBR RNAi* seedlings when treated with TSA (Supplementary Figure 3M-N), reminiscent to *fas RBR RNAi* seedlings, although more variable. Furthermore, additional layers of differentiated columella cells are induced by TSA treatment of *RBR*

RNAi seedlings (Supplementary Figure 3M-N). This suggests that epigenetic defects induced by inhibition of HDACs, cause overproliferation in the *RBR RNAi* background.

The CAF-1 complex acts in the SHR/SCR pathway and independent of the PLT pathway

To examine whether the CAF-1 complex acts in the PLT pathway, both *fas1-1* and *fas2-1* were crossed to *plt1-4 plt2-2* double mutants (Aida et al., 2004). Root size (Supplementary Figure 4A), meristem size (Figure 4A-C) and number of cortex cells (Supplementary Figure 4D-E; *t*-test, $P = 4\ 10^{-3}$, *fas1-1* ; $P = 7\ 10^{-4}$, *fas2-1*) of *fas plt1-4 plt2-2* triple mutant seedlings are smaller than *plt1-4 plt2-2* double mutants. Overexpression of *PLT2* induces an enlarged meristem in wildtype as described previously (Galinha et al., 2007). However, the stem cell defect is not rescued and meristem size is only transiently enlarged by overexpression of *PLT2* in both *fas1-1* and *fas2-1* mutants 10 dpg (Figure 4D-F). Together, this confirms that the CAF-1 complex acts independent of the PLT pathway.

Next, we investigated whether the CAF-1 complex acts in the SHR and SCR pathway. Both *fas scr-4* (Figure 4G-I; Supplementary Figure 4B; data not shown) and *fas shr-2* (Figure 4J-L; Supplementary Figure 4C; data not shown) double mutants show similar root size and meristem size as *scr-4* and *shr-2* single mutants. Strikingly, a homozygous population of *fas scr-4* double mutants could not be obtained, although *fas2-1 scr-4* homozygous seedlings can be found in a *fas2-1*$^{-/-}$ *scr-4*$^{+/-}$ population in the expected ratio (27%, n=15). This suggests that *fas scr-4* double mutants are defective in later stages of development and do not produce viable seeds. Similarly, a homozygous population of *fas shr-2* double mutants could not be obtained and homozygous seedlings are present 7 dpg in a *fas1-1*$^{-/-}$ *shr-2*$^{+/-}$ population in an aberrant ratio (12,5%, n=48). This suggests that the *fas shr-2* double combination affects gamete formation. Our data indicate that the CAF-1 complex acts in the SHR/SCR pathway for stem cell maintenance, even though it does not regulate *SHR* and *SCR* expression and localization. Lastly, in reproductive tissues a genetic interaction occurs between the CAF-1 complex and SHR or SCR.

The CAF-1 complex acts in a pathway partially overlapping with RBR

To confirm that the CAF-1 complex and RBR act independent of the PLT pathway, we analysed *fas RBR RNAi plt1-4 plt2-2* seedlings. *RBR RNAi* is unable to bypass PLT function (Wildwater et al., 2005), indicating that input from the PLT pathway is needed independent of RBR. *fas RBR RNAi plt1-4 plt2-2* seedlings have a smaller root size (Supplementary Figure 4F), differentiate faster than *plt1-4 plt2-2* seedlings, and do not show stem cell proliferation (Supplementary Figure 4G-I). In addition, overexpression of *PLT2* in *fas RBR RNAi* seedlings only transiently induces an enlarged meristem and reveals a disorganised cap like *fas RBR RNAi* (Figure 4P-R). Together, this shows that the CAF-1 complex and RBR act independently of PLT.

RBR RNAi is able to bypass *scr-4* differentiation (Wildwater et al., 2005) and therefore RBR formally acts downstream of SCR. To further investigate whether SCR, RBR and the CAF-1 complex act in the same pathway, we analysed *fas RBR RNAi scr-4* seedlings. These seedlings do not possess additional cell layers in the proximal meristem 3 dpg compared to *fas RBR RNAi* seedlings (Figure 4M-O) and have a similar root and meristem size (data not shown). This indicates that the CAF-1 complex and RBR have overlapping targets and that they act downstream of SCR.

Discussion

Here, we show that the CAF-1 complex and RBR synergistically repress root stem cell proliferation downstream of SCR action. Accordingly, the CAF-1 complex is involved in proliferation in leaves and hypocotyls (Exner et al., 2006; Ramirez-Parra and Gutierrez, 2007a). The expression domain of stem cell niche transcription factors *PLT*, *WOX5* and *FEZ* is expanded, while *SCR* expression disappears from the stem cell niche area in *fas RBR RNAi* seedlings, indicating an enlargement and repositioning of the stem cell niche. The enlargement of the stem cell niche may cause the occasionally observed splitting of the root tip in two separate tips. The altered localization of stem cell niche transcription factors in *fas RBR RNAi* seedlings resembles the dynamic changes occurring upon QC ablation and respecification (Xu et al., 2006). In the absence of the CAF-1 complex, repatterning of the stem cell niche probably occurs due to loss of QC specification. However, QC respecification is incomplete and stem cell overproliferation takes place in *fas RBR RNAi* seedlings, likely due to a failure in stem cell control.

The synergistic interaction between the CAF-1 complex and RBR indicates that they repress overlapping targets involved in proliferation and stem cell factors (like PLT1 and WOX5) in differentiating daughter cells.

We provide evidence that the CAF-1 complex and RBR synergistically promote correct differentiation. Our data are consistent with the notion that the *Arabidopsis* CAF-1 complex promotes correct trichome differentiation (Exner et al., 2006; Ramirez-Parra and Gutierrez, 2007a) and that *RBR* reduction represses differentiation in the root (Wildwater et al., 2005). In mammals, RB interacts with tissue-specific transcription factors and is known to facilitate differentiation (reviewed by Skapek et al., 2006). In analogy, the *Arabidopsis* CAF-1 complex and RBR might regulate tissue-specific transcription factors to promote differentiation.

Mutation of the *Arabidopsis* CAF-1 complex leads to activation of the DNA damage checkpoint and a switch to endoreduplication to overcome irreversible cell cycle arrest (Ramirez-Parra and Gutierrez, 2007a; Schonrock et al., 2006). *RBR* reduction does not directly affect cell cycle progression in the root (Wildwater et al., 2005). In mammalian cells, RB is a downstream target of the DNA damage response and mediates cell cycle arrest. In the absence of RB, cell cycle arrest is relieved although DNA damage is present (reviewed by Genovese et al., 2006). Therefore, reduction of *RBR* might lead to cell cycle progression in the *fas* mutant background, bypassing the DNA damage checkpoint which is normally activated. In this way, proliferation may be enhanced in *fas RBR RNAi* seedlings despite the activation of the checkpoint and the accumulation of (epigenetic) defects.

We show that mutation of the CAF-1 complex affects stem cell niche maintenance independent of *SCR*, *SHR* and *PLT* expression. The aberrant *SCR* expression as previously reported by Kaya et al. (2001) appears to be a late defect and is not correlated to the early stem cell niche defect in the primary root. Neither acute deletion of *FAS2* nor DNA damage leads to rapid stem cell niche misspecification, whereas the DNA polymerase inhibitor aphidicolin causes stem cell loss. This suggests that the stem cell defect in *fas* mutants is caused by an accumulation of defects due to stalling of the replication fork. Our data indicate that the CAF-1 complex acts genetically in the SHR/SCR/RBR pathway important for stem cell maintenance and independent of the PLT pathway.

Interestingly, we find that the stem cells are most sensitive to reduction of the CAF-1 complex and RBR action. Similarly, the mammalian CAF-1 complex is essential for maintenance of epigenetic marks in heterochromatin and correct heterochromatin compaction specifically in mouse ES cells (Houlard et al., 2006). In *Arabidopsis fas* mutants, heterochromatin compaction is reduced, although histone modifications in heterochromatin are not affected globally (Ramirez-Parra and Gutierrez, 2007a; Schonrock et al., 2006). In the future, the effect of the *Arabidopsis* CAF-1 complex on local heterochromatin compaction of stem cells needs to be investigated.

Previously it has been shown that histone chaperone MSI1 interacts with RB, HDACs and the CAF-1 complex in both animals and plants (reviewed by Hennig et al., 2005). However, it is unknown whether RB and CAF-1 are present in the same complex. In human fibroblasts, RB and the CAF-1 complex are present together at sites of DNA replication in early S-phase (Kennedy et al., 2000). We found a direct interaction in the yeast two-hybrid system between FAS1 and RBR and our data show that reduction of MSI1 has not a large effect. Therefore, MSI1 may not be necessary for the interaction between the CAF-1 complex and RBR, as they can interact directly.

The CAF-1 complex is thought to facilitate the correct inheritance of chromatin states through recruitment of several chromatin factors to the replication fork in mammals (reviewed by Groth et al., 2007; Wallace and Orr-Weaver, 2005). The same is true in *Arabidopsis*, since a small subset of genes in the *fas1* mutant are enriched for active histone modifications (acetylation) and deprived of a repressive histone modification (H3K9me2) (Ramirez-Parra and Gutierrez, 2007a). RB is known to be associated with many chromatin factors, including HDACs (reviewed by Frolov and Dyson, 2004; Giacinti and Giordano, 2006). Therefore, the *Arabidopsis* CAF-1 complex and RBR may together ensure propagation of repressive epigenetic marks at overlapping target genes. This is further supported by our finding that acute reduction of *RBR* in the *fas* mutant background as well as DNA stress treatments on *RBR RNAi* seedlings do not phenocopy *fas RBR RNAi* seedlings. Overproliferation does occur in *RBR RNAi* seedlings upon inhibition of HDACs by TSA, known to cause global hyperacetylation (Xu et al., 2005). Together, these data indicate that HDAC activity and RBR synergistically repress proliferation and that overproliferation is caused by an accumulation of epigenetic defects during successive cell divisions. The responsible HDAC is not HD1 alone (Tian et al., 2003) (NK, unpublished data) and other or

redundant HDACs might be involved. In mammalian cells, the CAF-1 complex incorporates newly synthesized histones acetylated at H4K5 and H4K12 and remains associated with heterochromatin for about 20 minutes, followed by a deacetylation event (Taddei et al., 1999). Interestingly, TSA prevents deacetylation of H4K5 and H4K12 when applied at the time of replication (Taddei et al., 1999). This suggests that the CAF-1 complex recruits a HDAC activity to ensure proper heterochromatin maturation and this might explain the similar synergistic effects we find between the CAF-1 complex or HDAC activity and RBR. In the future, the stem cell target sites of RBR and the CAF-1 complex, and the responsible HDACs need to be found to substantiate molecular mechanisms of epigenetic stem cell control.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotypes Columbia (Col), Landsberg *erecta* (L*er*), Enkheim (En) and Wassilewskija (WS) were used. *fas1-1* (En), *fas2-1* (L*er*) and *fas2-4* (Col, SALK_033228, T-DNA insertion in the 6th exon) were obtained from the Nottingham *Arabidopsis* Stock Centre. *fas1-1* three times backcrossed to Col and *msi1-as* seeds (Col) (Exner et al., 2006) were kindly provided by L. Hennig.
Origins and ecotypes of other markers and mutants are as follows: QC25 (WS) (Sabatini et al., 2003); *pSHR::GFP:SHR* (Col) (Nakajima et al., 2001); *35S::PLT2:GR* (Col) (Galinha et al., 2007); *DR5::GUS* (Col) (Ulmasov et al., 1997); *cyclinB1;1:GUS* (Col) (Colon-Carmona et al., 1999); *scr-4* (WS), *shr-2* (Col) (Helariutta et al., 2000); *RBR RNAi* (Col) (Wildwater et al., 2005); *plt1-4 plt2-2* (WS) (Aida et al., 2004).

Origin and concentration of DNA stress agents are as follows: aphidicolin (12μg/ml, Sigma), hydroxyurea (5mM, Sigma), MMS (methyl methanesulfonate; 0.01%, Sigma), zeocin (10μg/ml, Duchefa). Histone deacetylase inhibitor TSA (Trichostatin A) was obtained from Sigma, dissolved in dimethylsulfoxide (stock concentration 5 mM), applied upon germination (1μM TSA) and stored at -20^{o}C until use. Different concentrations of the agents were tested and the lowest concentration with apparent phenotypes was used.
Plants were grown as described before (Sabatini et al., 1999).

Transgenic plants

For the construction of *FAS2* deletion clones, a 7 kb XhoI genomic fragment containing the *FAS2* gene and 3 kb of its promoter was isolated from BAC Miub3 (obtained from ABRC) and was cloned into the *pCB1* vector (Heidstra et al., 2004). The resulting *pCB1-FAS2* vector was transformed into *fas2-1* mutant plants. The *pG7HSCRE* vector (Heidstra et al., 2004) was crossed into the *fas2-1* mutant background. *fas2-1* plants containing the *pCB1-FAS2* vector were crossed with plants containing the *pG7HSCRE* vector, after which the resulting F1 generation was heat-shocked and analysed.

For the construction of *pSCR::GFP*, a 2.4 kb HindIII fragment of the *SCR* promoter was generated by PCR using primers pSCRF (5'-TCTCTATGAAAAGTGGAAATTTACCT-GGAA-3') and pSCRR (5'-GGAGATTGAAGGGTTGTTGGTCGTG-3') and cloned into *pGII226-GFP$_{ER}$T*.
Transgenic plants were generated using the floral dip method (Clough and Bent, 1998).

Microscopy and in situ hybridization

Light microscopy and fluorescence microscopy was performed as described (Willemsen et al., 1998). Starch granules and β-glucuronidase activity were visualised as before (Willemsen et al., 1998). Aniline blue staining of mature embryos was performed according to (Bougourd et al., 2000). Root length, meristem size and differentiated epidermis cell size was determined as described (Willemsen et al., 1998). Whole-mount in situ hybridisation was performed manually (Hejatko et al., 2006) using *PLT1* probe, *WOX5* probe and *FEZ* probe (whole cDNA) as described (Aida et al., 2004; Sarkar et al., 2007; Willemsen et al., submitted).

Yeast two-hybrid assay

Yeast two-hybrid interactions were studied using the ProQuest Two Hybrid System (Invitrogen Life Technologies). The full length cDNAs of *RBR* and *FAS1* were amplified using primers RBRc1F (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCttggatccat-ggaagaagttcagcctccagtgac-3') and RBRc2R (5'-GGGGACCACTTTGTACAAG-AAAGCTGGGTtctatgaatctgttggctcggttttaagg-3') for *RBR* and primers fas1FW (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTatggacgaagtttcgacggtgaat-3') and fas1RV

(5'-GGGGACCACTTTGTACAAGAAAGCTGGGTttaagcattttcgttttccaatctct-3') for *FAS1* and were then fused to both the *pDEST32 BD* and *pDEST22 AD* vectors. Construction of *SHR* and *SCR* vectors for yeast two-hybrid were as described before (Welch et al., 2007). Autoactivation of yeast containing bait was tested using selective medium – His – Leu and supplied with 10 mM 3AT (3-Amino-1,2,4-Triazol, Fluka). The bait and pray constructs were transformed into the yeast strains Pj694α and Pj694a, respectively. Mating and selection were performed as described (James et al., 1996).

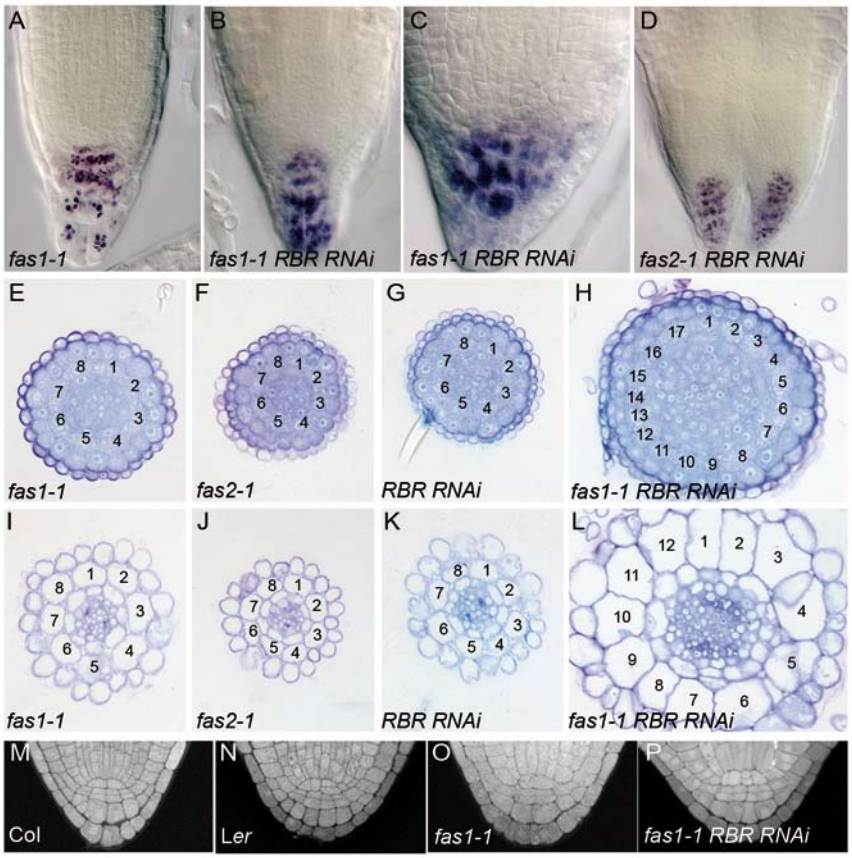
Acknowledgements

L. Hennig kindly provided *fas1-1* three times backcrossed to Col and *msi1-as* seeds. We thank R. Leito and F. Kindt for image processing.

Supplementary Table 1 Percentage of mature embryos with QC divisions as determined by aniline blue staining

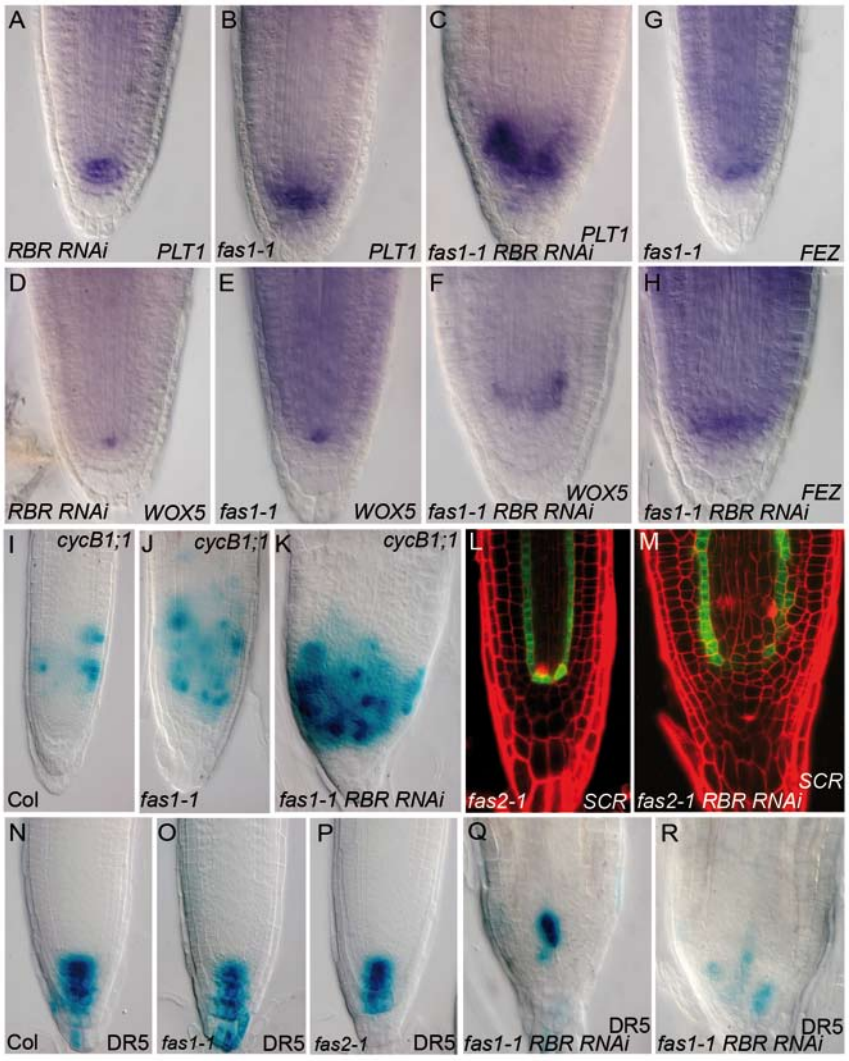
	QC division (%)	total number
Col	8	38
En	0	35
L*er*	45	33
fas1-1	22	46
fas2-1	24	46
RBR RNAi	3	35
fas1-1 RBR RNAi	70	46
fas2-1 RBR RNAi	55	56

Supplementary Figure 1 The CAF-1 complex and RBR synergistically repress stem cell proliferation



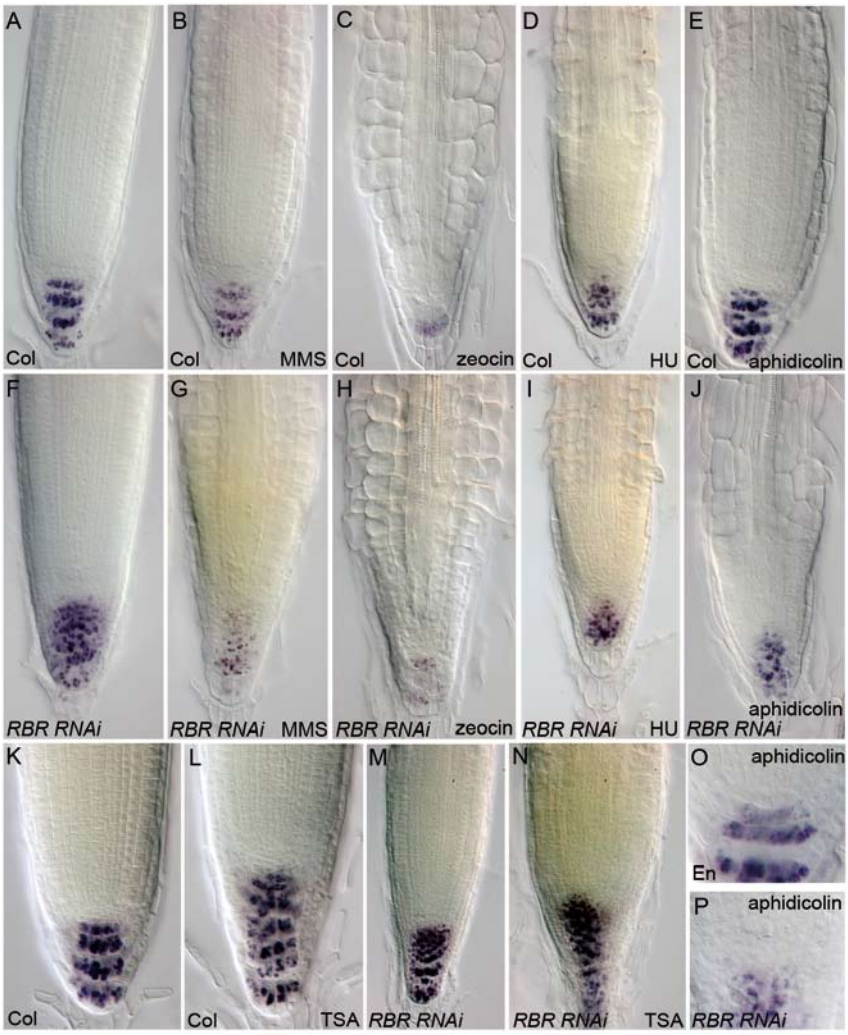
(A-D) Root tip of *fas1-1* (A) 5 dpg, *fas1-1 RBR RNAi* (B) 5 dpg, *fas1-1 RBR RNAi* (C) 3 dpg, *fas2-1 RBR RNAi* (D) 7 dpg. (E-L) Cross section just above the stem cells (E-H) or in the elongation zone (I-L) of *fas1-1* (E,I), *fas2-1* (F,J), *RBR RNAi* (G,K) and *fas1-1 RBR RNAi* (H,L) seedlings, 3 dpg. (M-P) Analine blue staining of mature Col (M), L*er* (N) and *fas1-1* (O) and *fas1-1 RBR RNAi* (P) embryos.

Supplementary Figure 2 The CAF-1 complex and RBR synergistically regulate expression of stem cell niche transcription factors



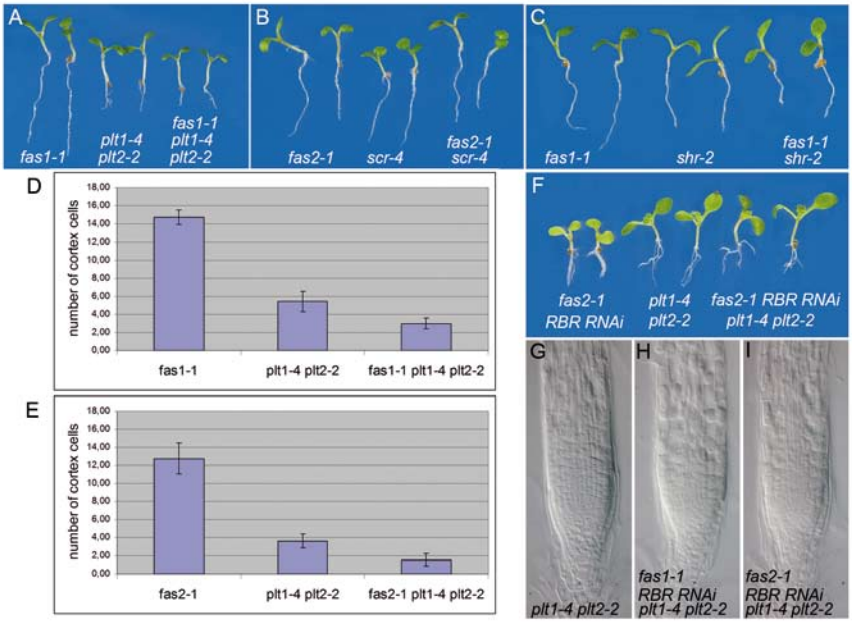
(A-H) In situ hybridisation using *PLT1* probe (A-C), *WOX5* probe (D-F) and *FEZ* probe (G-H) in *RBR RNAi* (A,D), *fas1-1* (B,E,G) and *fas1-1 RBR RNAi* (C,F,H), 2 dpg. (I-K) Expression of *cycB1;1:GUS* in Col (I), *fas1-1* (J), *fas1-1 RBR RNAi* (K), 4 dpg. (L-M) Expression of *pSCR::GFP* in *fas2-1* (L) and *fas2-1 RBR RNAi* (M), 4 dpg. (N-R) Expression of *DR5::GUS* in Col (N), *fas1-1* (O), *fas2-1* (P) 7 dpg, and *fas1-1 RBR RNAi* (Q-R) 4 dpg.

Supplementary Figure 3 DNA stress does not cause overproliferation in *RBR RNAi* seedlings, whereas inhibition of HDACs by TSA does



(A-J,O-P) Root tip of Col (A-E), En (O) and *RBR RNAi* (F-J,P), without (A,F), 2 days after treatment with 0.01% MMS (B,G), 2 days after treatment with 10μg/ml zeocin (C,H), 3 days after treatment with 5mM hydroxyurea (HU) (D,I), germinated on 12μg/ml aphidicolin (E-J,O-P), 7 dpg. Note stem cell loss in En (O) treated with aphidicolin. (K-N) Root tip of Col (K-L) and *RBR RNAi* (M-N), without (K,M) or germinated on 1μM TSA (L,N), 7 dpg.

Supplementary Figure 4 The CAF-1 complex and RBR act in the SHR/SCR pathway and independent of the PLT pathway



(A-C) Root length of *fas1-1 plt1-4 plt2-2* and singles (A), *fas2-1 scr-4* and singles (B), *fas1-1 shr-2* and singles (C), 7 dpg. (D-E) Quantification number of cortex cells of *fas1-1 plt1-4 plt2-2* (D) and *fas2-1 plt1-4 plt2-2* (E), 4 dpg. Error bars indicate 95% confidence interval. (F) Root length of *fas2-1 RBR RNAi plt1-4 plt2-2* and doubles, 7 dpg. (G-I) Root tip of *plt1-4 plt2-2* (G), *fas1-1 RBR RNAi plt1-4 plt2-2* (H) and *fas2-1 RBR RNAi plt1-4 plt2-2* (I), 7 dpg.

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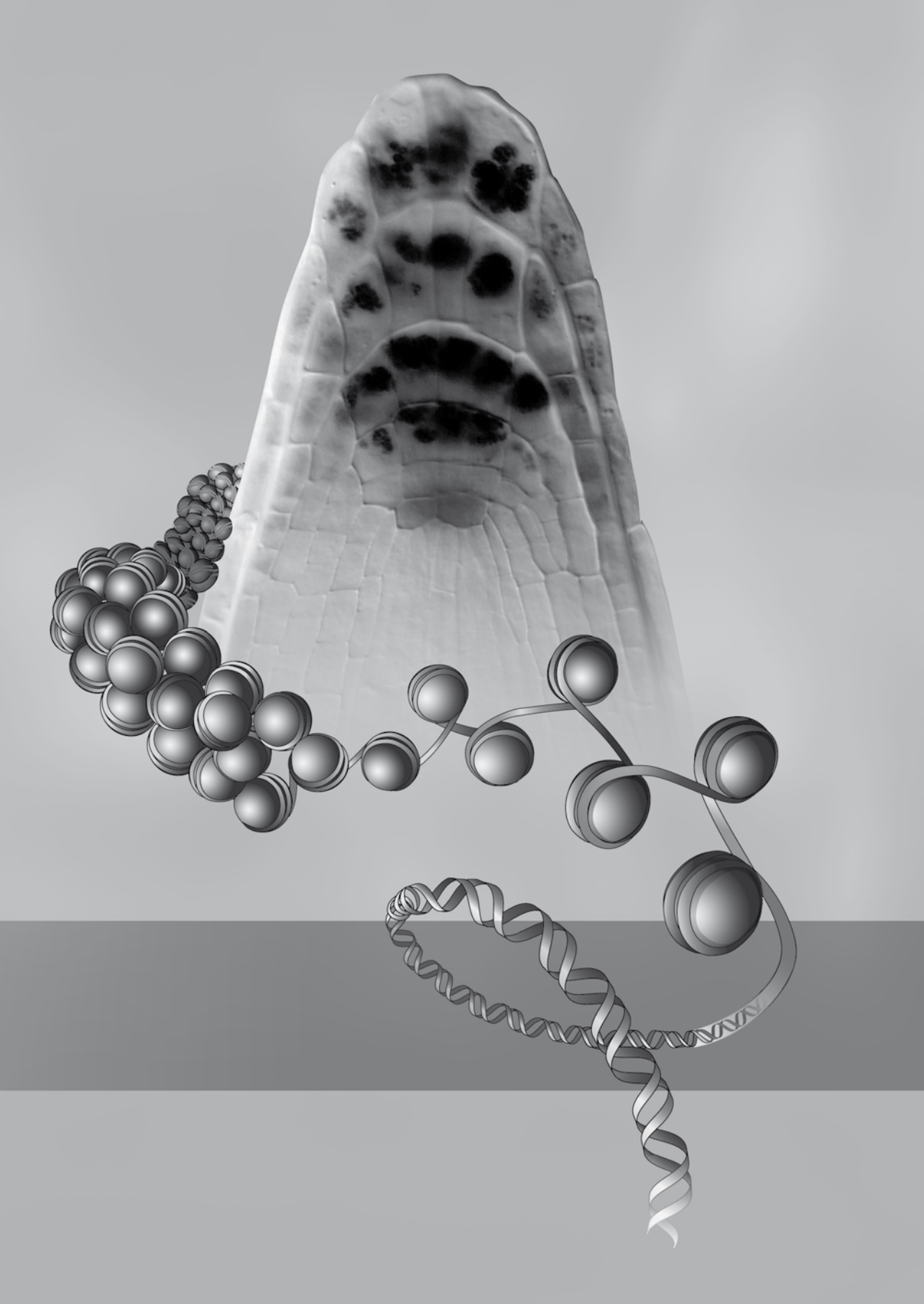


Arabidopsis Polycomb group genes are required for root stem cell maintenance

CHAPTER 4

Noor Kornet and Ben Scheres

Summary

Polycomb group complexes are well known to regulate the expression of key developmental regulators. Recent data in mammals provide evidence that PRC2 Polycomb proteins prevent differentiation in ES cells. Here, we show that *Arabidopsis* PRC2 Polycomb genes *SWINGER (SWN*) and *CURLY LEAF (CLF)* are necessary for root stem cell maintenance. In addition, expression of the stem cell transcription factor *SCR* is stochastically lost early during root development. Our data indicate that SWN and CLF act in the SCR pathway and are redundantly required with RETINOBLASTOMA RELATED (RBR) to stimulate proliferation or prevent differentiation of the transit amplifying cells.

Introduction

Polycomb group proteins are known to repress transcription of developmental genes, e.g. fly and mammalian Hox genes, and provide cellular memory of gene expression states (reviewed by Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007). In animals, the Polycomb repressive complex 2 (PRC2) consists of E(z), ESC and Su(Z)12, with E(z) possessing H3K27 trimethylation activity. The PRC1 complex is recruited to sites of H3K27me3 and represses transcription (Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007).

In *Arabidopsis*, duplicated PRC2 complexes are involved in different aspects of plant development (Pien and Grossniklaus, 2007; Schubert et al., 2005), whereas there are no homologues of a PRC1 complex. The MEDEA (MEA), FERTILISATION INDEPENDENT ENDOSPERM (FIE), FERTILISATION INDEPENDENT SEED2 (FIS2) PRC2 complex is involved in imprinting during endosperm development and represses, for example, the MADS box transcription factor *PHERES1* (Kohler et al., 2003; Makarevich et al., 2006). Disruption of the MEA PRC2 complex causes endosperm proliferation without fertilisation (reviewed by Autran et al., 2005; Huh et al., 2007; Kohler and Makarevich, 2006).

While the MEA PRC2 complex is mainly required to control seed development, the other two *Arabidopsis* PRC2 complexes play roles during embryonic, vegetative and flower development. The CURLY LEAF (CLF)/ SWINGER (SWN), FIE, VERNALIZATION2 (VRN2) PRC2 complex represses, for example, the floral repressor *FLC* upon vernalization and induces flowering (Bastow et al., 2004; Gendall et al., 2001). Furthermore, the CLF/SWN, FIE, EMBRYONIC FLOWER2 (EMF2) PRC2 complex is required to repress the floral homeotic genes *AGAMOUS (AG)* and *APETELA3 (AP3)* during embryonic and vegetative development (Goodrich et al., 1997; Hennig et al., 2003; Katz et al., 2004; Kinoshita et al., 2001; Schubert et al., 2006).

Like their animal counterparts, E(z) homologues MEA, SWN and CLF are implicated in the deposition of H3K27me2/3 marks (Bastow et al., 2004; Lindroth et al., 2004; Makarevich et al., 2006; Schonrock et al., 2006; Schubert et al., 2006; Sung and Amasino, 2004).

EMF2 and VRN2, and CLF and SWN are partially redundant and double mutants have a more severe phenotype (Chanvivattana et al., 2004; Lindroth et al., 2004; Makarevich et al., 2006; Schubert et al., 2006). These include aberrant development of the shoot and root to shoot transformations (Schubert et al., 2005). Similar phenotypes are found when the *fie* mutant is rescued through seed development ("rescued *fie*") (Kinoshita et al., 2001).

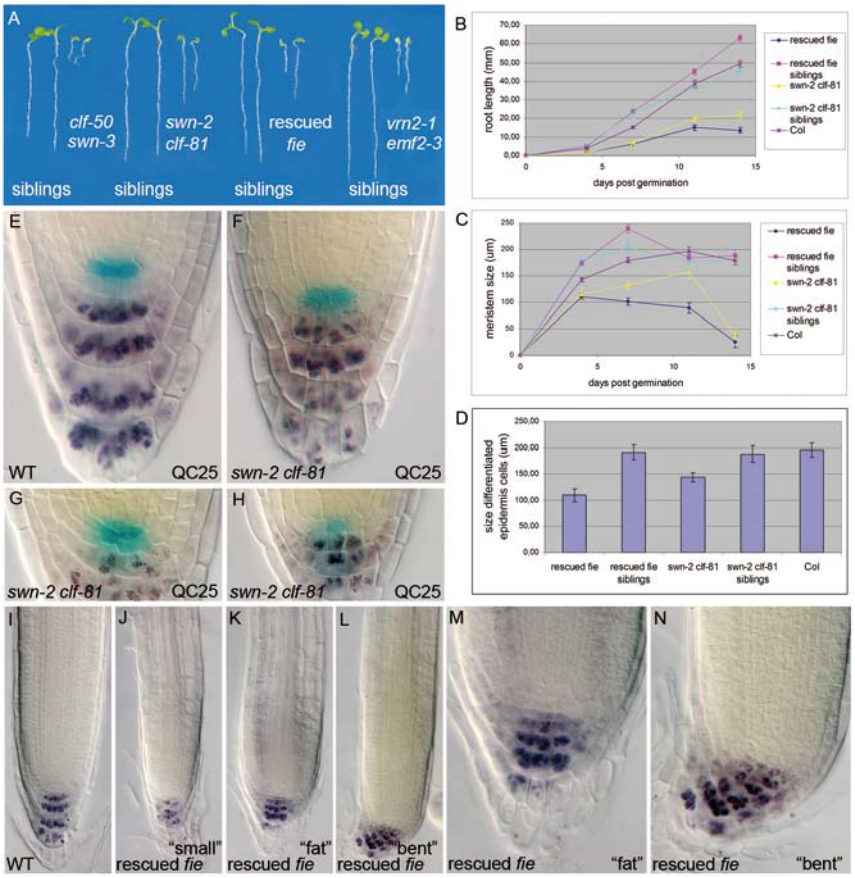
Here, we show that *Arabidopsis* PRC2 complexes are involved in root stem cell function. SWN and CLF are redundantly required to maintain the stem cells and expression of the root stem cell transcription factor *SCARECROW (SCR)* (Sabatini et al., 2003). Loss of stem cells and *SCR* expression in *swn-2 clf-81* seedlings occurs before the root to shoot transformation. Reduction of *RETINOBLASTOMA RELATED (RBR)* leads to additional stem cell layers and is able to bypass mutation of *SCR* (Wildwater et al., 2005). Differentiation of *swn-2 clf-81* seedlings is not rescued by *RBR RNAi*, but instead differentiation is enhanced by reduction of *RBR* in *swn-2 clf-81* seedlings. This shows that RBR is redundantly required with SWN and CLF to stimulate proliferation or prevent differentiation of the transit amplifying cells.

Results

Polycomb group genes are required for stem cell maintenance

To investigate in detail the role of Polycomb group genes in root development, we obtained *swn-2 clf-81*, *clf-50 swn-3*, *vrn2-1 emf2-3* and rescued *fie* mutants (see materials and methods). Root length is shorter in all mutants (Figure 1A-B), and the meristem is not maintained and differentiates at 14 dpg (Figure 1C; data not shown). The size of differentiated epidermis cells is smaller in *swn-2 clf-81* and rescued *fie* mutants (Figure 1D). Previously, it was reported that the root transforms into a shoot after four to five weeks (Chanvivattana et al., 2004; Kinoshita et al., 2001). At 10 dpg only 6% of *vrn2-1 emf2-3* seedlings contain a green root tip (Table 1), whereas this is not found in *swn-2 clf-81* and rescued *fie* mutants 10 dpg (Table 1). This indicates that homeotic transformations occur at a later time-point. At 7 dpg, most of the rescued *fie* roots have a smaller meristem (Figure 1J; Table 1), "fat" roots (Figure 1K,M) and roots with root cap bending (Figure 1L,N) are present in lower frequencies (Table 1). The latter phenotype is similar to the root cap bending we observed in *FAS2* and *RBR*

Figure 1 Root phenotype of *Arabidopsis* PRC2 Polycomb complex mutants



(A-D) Phenotype (A), root length (B), meristem size (C) and size of differentiated epidermis cells (D) of *clf-50 swn-3, swn-2 clf-81*, rescued *fie* and *vrn2-1 emf2-3* seedlings, 7 dpg. Double mutants were selected from a population deriving from *clf-50*$^{-/-}$ *swn-3*$^{+/-}$, *swn-2*$^{-/-}$ *clf-81*$^{+/-}$, and *vrn2-1*$^{-/-}$ *emf2-3*$^{+/-}$ plants. Siblings represent seedlings homozygous for one allele and wildtype or heterozygous for the other allele. Error bars indicate 95% confidence interval. (E-H) QC25 expression and starch granules in wildtype (E) and *swn-2 clf-81* (F-H), 4 dpg (E-F) and 7 dpg (G-H). (I-N) Root tip of wildtype (I) and rescued *fie* mutant (J-N), with small meristem (J), "fat" root (K-M) and root cap bending (L-N), 7 dpg.

deletion clones (Chapter 3; Wildwater et al., 2005). "Fat" roots are radially expanded and seem to have embryonic-like properties. The frequency of "fat" roots and roots with root cap bending is lower in *swn-2 clf-81* mutants (Table 1).

Table 1 Frequencies PRC2 Polycomb mutant phenotypes

	swn-2 clf-81	**rescued *fie***	***vrn2-1 emf2-3***
root-shoot	0%	0%	6%
small meristem	85%	70%	nd
"fat" roots	14%	23%	nd
cap bending	1%	7%	nd

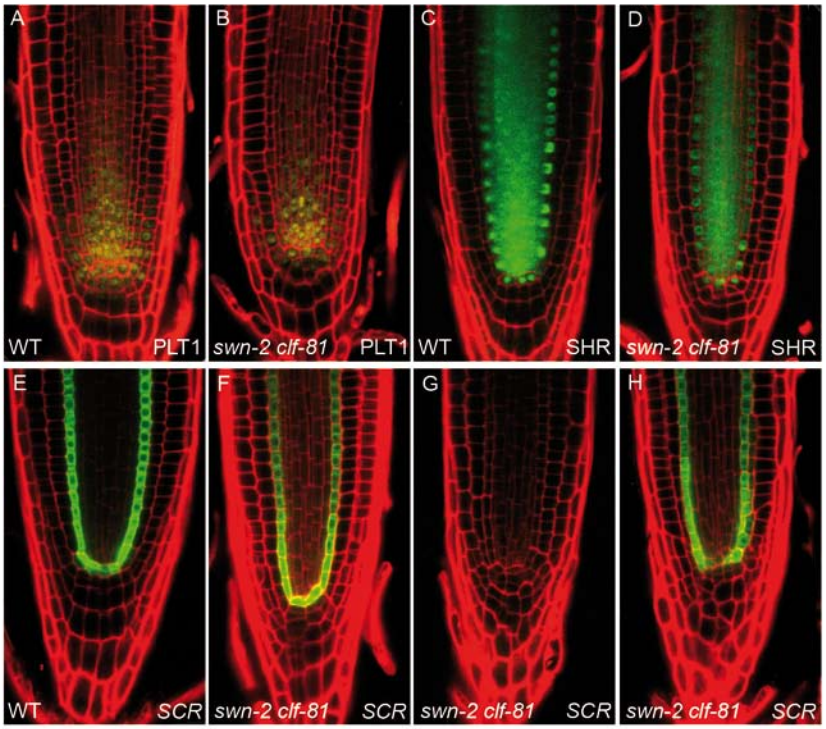
Root-shoot, root to shoot transformations, 10 dpg. Root phenotypes 7 dpg: small meristem (Figure 1J), embryonic like (Figure 1 K,M) and bending of the root cap (Figure 1L,N). *swn-2 clf-81*, n=35; rescued *fie,* n=30; *vrn2-1 emf2-3*, n=35; nd, not done.

Root growth is maintained by the organising centre or quiescent centre (QC) and its surrounding stem cells (van den Berg et al., 1995). To investigate whether the stem cell niche is affected in the *swn-2 clf-81* mutant, it was crossed to a QC specific marker (QC25). In wildtype, a layer of columella stem cells is present between the QC and differentiated columella cells (which contain starch granules) (Figure 1E). In contrast, *swn-2 clf-81* mutants display starch granules next to the QC 4 dpg (Figure 1F; 47%, n=47) and 7 dpg (Figure 1G-H; 45%, n=20), while they are present 3 dpg (data not shown). This shows that stem cell loss is an early event caused by mutation of *SWN* and *CLF.*

SWN and CLF are necessary to maintain *SCR* expression

The root stem cell niche is defined by the PLETHORA (PLT) pathway (Aida et al., 2004; Blilou et al., 2005) and the SHORTROOT (SHR)/ SCR pathway (Helariutta et al., 2000; Nakajima et al., 2001; Sabatini et al., 2003). To determine whether the stem cell defect in the *swn-2 clf-81* mutants is caused by misregulation of these stem cell niche transcription factors, we analysed their expression. Expression of a translational fusion of *PLT1* (Galinha et al., 2007) is not affected in *swn-2 clf-81* roots 7 dpg (Figure 2A,B), indicating that the stem cell defect is independent of the PLT pathway. Similarly, localization and expression of SHR is not changed in *swn-2 clf-81* roots 6 dpg (Figure 2C,D). Expression of *SCR* is normal in 40% (n=42) of *swn-2 clf-81* roots 4 dpg (Figure 2F). However, expression of *SCR* is completely absent in 60% (n=42) of *swn-2 clf-81* roots 4 dpg (Figure 2G). The percentage of roots without *SCR* expression is higher

Figure 2 Expression of stem cell transcription factors in *swn-2 clf-81* mutant



(A-D) Expression of *PLT1* (A-B), and *SHR* (C-D) translational fusion in wildtype (A,C) and *swn-2 clf-81* (B,D), 7 dpg (A-B) and 6 dpg (C-D). (E-H) Expression of *SCR* transcriptional fusion in wildtype (E) and *swn-2 clf-81* (F-H), 6 dpg (E-G) and 7 dpg (H).

(77%, n=53) at 7 dpg. In addition, aberrant expression of *SCR* in the stem cell niche area can be occasionally observed at 7 dpg (Figure 2H). This indicates that SWN and CLF are required redundantly to maintain expression of *SCR*.

RBR is redundantly required with SWN and CLF to stimulate proliferation or prevent differentiation

Reduction of *RBR* results in additional stem cell layers in the root (Figure 3D). *RBR RNAi* is able to bypass *scr* differentiation and therefore RBR formally acts downstream

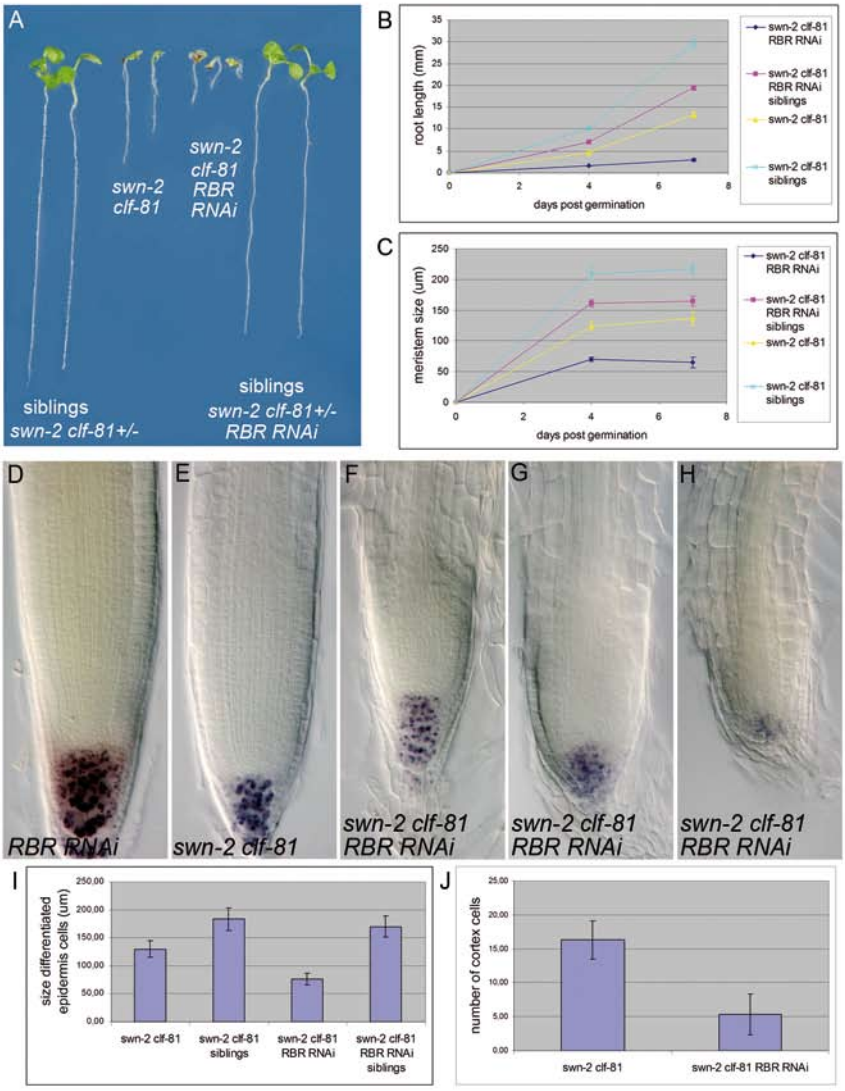
of SCR (Wildwater et al., 2005). We investigated whether reduction of *RBR* can rescue the stem cell defect of *swn-2 clf-81* mutants. *swn-2 clf-81 RBR RNAi* seedlings possess a smaller root (Figure 3A-B), meristem (Figure 3C-H) and number or cortex cells (Figure 3J) compared to *swn-2 clf-81* mutants. The size of differentiated epidermis cells is smaller in *swn-2 clf-81 RBR RNAi* seedlings (Figure 3I) and could partially cause the smaller root length. Additional columella layers are only transiently observed and not in all roots of *swn-2 clf-81 RBR RNAi* seedlings (Figure 3F-G), indicating that reduction of *RBR* can not rescue stem cell loss due to mutation of *SWN* and *CLF*. Interestingly, root (Figure 3A-B) and meristem size (Figure 3C) of siblings, which are *swn-2*$^{-/-}$ *RBR RNAi* and wildtype or heterozygous for the *clf-81* allele, is smaller compared to *swn-2*$^{-/-}$ *clf-81*$^{+/-}$ seedlings, whereas the size of differentiated epidermis cells is not affected (Figure 3I). This shows that reduction of *RBR* in *swn-2*$^{-/-}$ *clf-81*$^{+/-}$ seedlings causes enhanced differentiation, indicating a specific synergistic effect. These data uncover a role of RBR in stimulating proliferation or preventing differentiation in the proximal meristem. Together, our data show that SWN and CLF act downstream of RBR or in an independent pathway. In addition, RBR is redundantly required with SWN and CLF to stimulate proliferation or prevent differentiation.

Discussion

Here, we show that *Arabidopsis* PRC2 complexes are involved in stem cell control: SWN and CLF are redundantly required for root stem cell maintenance. In the animal field, PRC2 complexes are implicated in deposition of H3K27me3 at differentiation genes in mouse and human ES cells (Azuara et al., 2006; Boyer et al., 2006; Lee et al., 2006; Pasini et al., 2007). Similarly, the *Arabidopsis* CLF/SWN, FIE, EMF2, MSI1 PRC2 complex represses the expression of genes implicated in differentiation, like the floral homeotic genes *AGAMOUS* and *APETELA3* (Goodrich et al., 1997; Hennig et al., 2003; Katz et al., 2004; Kinoshita et al., 2001; Schubert et al., 2006). When the PRC2 complex is mutated in mammalian ES cells, differentiation genes are prematurely expressed. Therefore, root stem cell loss in *swn-2 clf-81* mutants might be caused by precocious expression of differentiation genes that remain to be identified.

Mutated PRC2 complexes have pleiotropic phenotypes, with severe defects in both shoot and root. Expression of stem cell transcription factors *PLT1* and *SHR* is unaltered

Figure 3 Enhanced differentiation in *swn-2 clf-81* mutants caused by reduction of *RBR*



(A-C) Phenotype (A), root length (B) and meristem size (C) of *swn-2 clf-81, swn-2 clf-81 RBR RNAi* and their siblings. Siblings represent seedlings homozygous for *swn-2* and *RBR RNAi*, and wildtype or heterozygous for the *clf-81* allele. Error bars indicate 95% confidence interval. (D-H) Root tip of *RBR RNAi* (D), *swn-2 clf-81* (E) and *swn-2 clf-81 RBR RNAi* (F-H), 7 dpg. (I-J) Size of differentiated epidermis cells (I) and number of cortex cells (J) of *swn-2 clf-81, swn-2 clf-81 RBR RNAi* and their siblings, 7 dpg.

in *swn-2 clf-81* seedlings, suggesting that the defects are not global. However, expression of *SCR* is stochastically lost and increases in time, indicating that SWN and CLF are redundantly required to maintain *SCR* expression. In addition, *swn-2 clf-81* seedlings differentiate at the same time as *scr* mutants (Sabatini et al., 2003), suggesting that SWN and CLF act in the SCR pathway. *SCR* is not a putative target of PRC2 complexes as H3K27me3 is not enriched (Zhang et al., 2007). SWN and CLF might be required to repress a negative regulator of *SCR*, which is stochastically released in the *swn-2 clf-81* mutant, causing loss of *SCR* expression.

Reduction of *RBR* is able to bypass *scr* differentiation (Wildwater et al., 2005) and a formal interpretation would be that RBR acts downstream of SCR. However, differentiation of *swn-2 clf-81* and *swn-2*$^{-/-}$ *clf-81*$^{+/-}$ seedlings is enhanced by *RBR RNAi*, indicating that SWN and CLF synergistically interact with RBR. These data reveal that RBR is redundantly required with SWN and CLF to stimulate proliferation or prevent differentiation of transit amplifying cells. Intriguingly, this is opposite to previous data that low levels of RBR delay differentiation and high levels induce differentiation (Wildwater et al., 2005). SWN, CLF and RBR could synergistically regulate specific target genes, involved in proliferation or differentiation. In mammalian fibroblasts, the PRC1 and PCR2 complexes bind to the *Ink4a*/*Arf* locus, encoding two cell cycle inhibitors (Lowe and Sherr, 2003; Sharpless, 2005), in a RB family dependent manner (Bracken et al., 2007; Kotake et al., 2007). This implicates that RB and Polycomb complexes together repress expression of *Ink4a* and thereby stimulate cell cycle progression. In *Arabidopsis*, RBR may interact directly with FIE (Mosquna et al., 2004) and SCR contributes to proliferation in the transit amplifying cells, independent of its role in stem cell maintenance (Sabatini et al., 2003). Therefore, RBR and *Arabidopsis* PRC2 complexes may together, downstream of SCR, control target genes involved in transit amplifying cell proliferation and this will be investigated in the future.

The mammalian key differentiation regulators not only contain repressive marks (H3K27me3) in ES cells, but also active marks (H3K4me2/3 and H3K9 acetylation), forming so called bivalent domains (reviewed by Buszczak and Spradling, 2006; Gan et al., 2007; Meshorer and Misteli, 2006). The current theory is that genes necessary for differentiation are repressed, while being poised for activation at the same time. Upon differentiation, the bivalent domains are lost and resolved into either active or repressive chromatin. Until now, the existence of bivalent domains in *Arabidopsis* has

not yet been reported, although Trithorax homologues exist (reviewed by Pien and Grossniklaus, 2007), e.g. ATX1 possesses H3K4 methyltransferase activity and promotes the expression of homeotic genes, including *AGAMOUS* (Alvarez-Venegas et al., 2003). Mammalian stem cell factors Oct4, Nanog and Sox2 are known to bind to the promoters of a large subset of differentiation genes and might direct the deposition of bivalent domains (Boyer et al., 2005; Lee et al., 2006). In the near future, target genes of the *Arabidopsis* stem cell transcription factors (PLT, SHR and SCR) will be identified. This should allow a molecular investigation of the role of PRC2 and Trithorax complexes in chromatin regulation in plant stem cells.

Materials and methods

Plant materials and growth conditions

Seeds of *swn-2 clf-81*, *clf-50 swn-3* (Chanvivattana et al., 2004) and *vrn2-1 emf2-3* were kindly provided by J. Goodrich. *swn-2* (Col) and *swn-3* (Col) contain a T-DNA insertion in the 9th intron (SALK_010213) and 15th exon (SALK_050195), respectively (Chanvivattana et al., 2004). *clf-81* (Col) possesses an amino acid substitution (R794H) (Schubert et al., 2006), *clf-50* (WS) is a deletion of the entire locus (Chanvivattana et al., 2004), *vrn2-1* (L*er*) has a stop codon in the 13th exon (Gendall et al., 2001) and *emf2-3* (Col) contains a 35 bp deletion in the 20th exon (Yoshida et al., 2001).

Rescued *fie* seeds (Kinoshita et al., 2001) were obtained from R. Fischer. In rescued *fie* plants seed viability is restored of *fie-1* mutants by a transgene, *pFIE::FIE:GFP*, which is only expressed in the central cell and during early endosperm development. *fie-1* contains a G to A substitution at the border of the 1st intron, probably causing a splicing defect.

Origins and ecotypes of other markers and mutants are as follows: QC25 (WS) (Sabatini et al., 2003); *pSHR::GFP:SHR* (Col) (Nakajima et al., 2001); *pPLT1::PLT1:YFP* (Col) (Galinha et al., 2007); *RBR RNAi* (Col) (Wildwater et al., 2005).
Plants were grown as described before (Sabatini et al., 1999).

Transgenic plants

For the construction of *pSCR::GFP,* a 2.4 kb HindIII fragment of the *SCR* promoter was generated by PCR using primers pSCRF (5'-TCTCTATGAAAAGTGGAAATTTACCT-GGAA-3') and pSCRR (5'-GGAGATTGAAGGGTTGTTGGTCGTG-3') and cloned into *pGII226-GFP$_{ER}$T*.
Transgenic plants were generated using the floral dip method (Clough and Bent, 1998).

Microscopy

Light microscopy and fluorescence microscopy was performed as described (Willemsen et al., 1998). Starch granules and β-glucuronidase activity were visualised as before (Willemsen et al., 1998). Root length, meristem size and differentiated epidermis cell size was determined as described (Willemsen et al., 1998).

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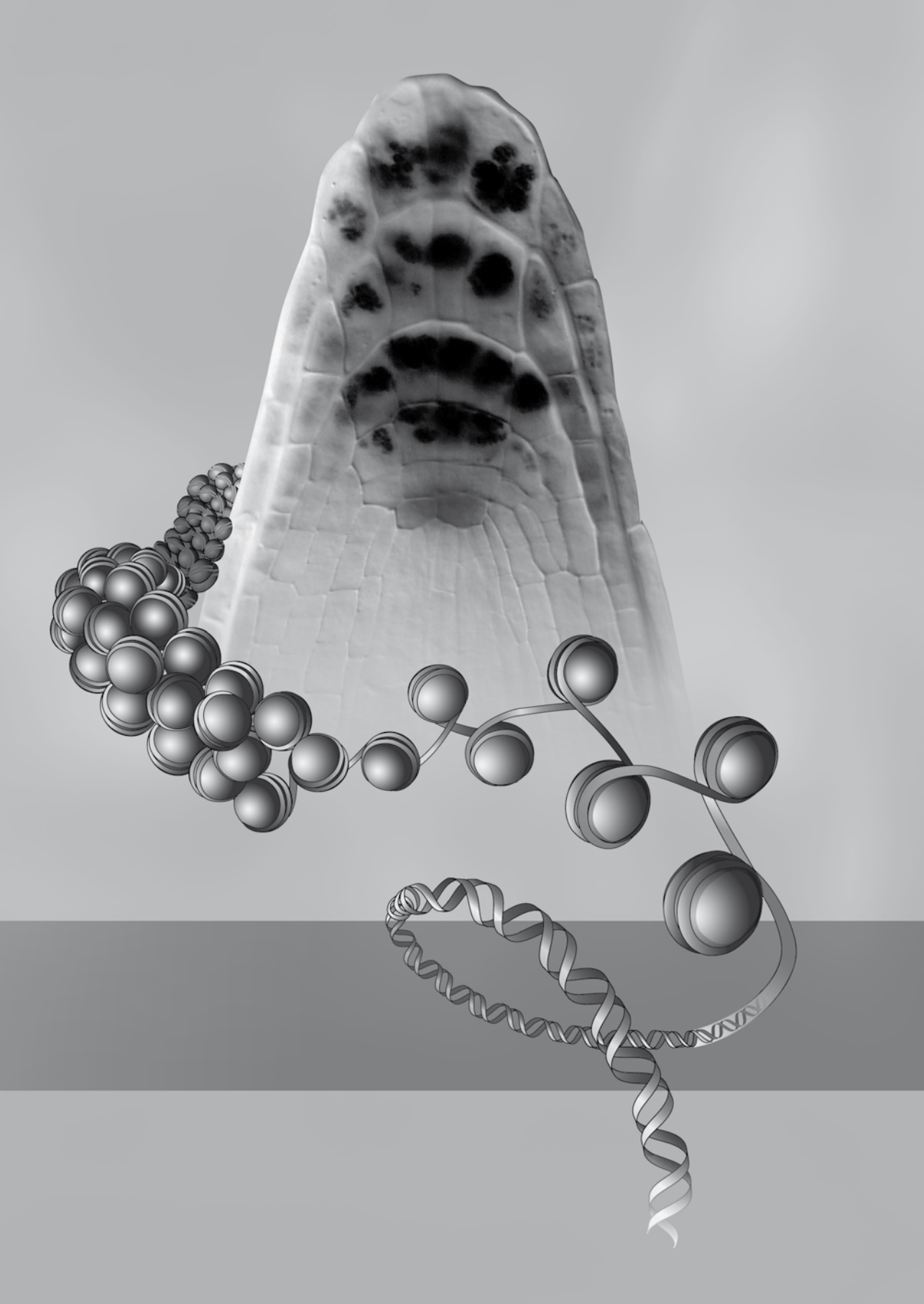


Summarizing discussion

CHAPTER 5

Summarizing discussion

Stem cells replenish the cells present in an organism throughout its lifetime and sustain growth. They have unique characteristics: the capability to self-renew and the potential to differentiate into several cell types. Recently, it has become clear that chromatin factors support these unique features in mammalian stem cells (**Chapter 1**) (reviewed by Jaenisch and Young, 2008; Niwa, 2007a; Niwa, 2007b; Spivakov and Fisher, 2007).

In this thesis, we describe investigations on the roles of chromatin factors in *Arabidopsis* root stem cell maintenance. We performed a genome-wide survey of chromatin factor mutants affecting histone acetyltransferases (HATs), histone deacetylases (HDACs), CAF-1 nucleosome assembly complex, PRC2 Polycomb complexes, remodellers and histone methyltransferase (HMT) proteins, to identify which classes of chromatin factors could be genetically linked to stem cell action. Next, we determined how such chromatin factors relate to the transcription factors already known to define the root stem cells: PLETHORA (PLT), SHORTROOT (SHR), and SCARECROW (SCR), and to RETINOBLASTOMA RELATED (RBR) (Aida et al., 2004; Blilou et al., 2005; Galinha et al., 2007; Helariutta et al., 2000; Nakajima et al., 2001; Sabatini et al., 2003; Wildwater et al., 2005).

We find that several classes of chromatin factors (HAT (GCN5), the CAF-1 complex, HDACs, PRC2 Polycomb proteins) regulate root stem cell niche maintenance. GCN5 acts in the PLT pathway and the CAF-1 complex, HDACs and PRC2 complex members act in the SHR/SCR/RBR pathway. This indicates that chromatin factors play a role in both parallel pathways known to define the root stem cells. Interestingly, chromatin factors associated with active chromatin are found in the PLT pathway, while chromatin factors required to maintain repressive chromatin are acting in the SHR/SCR/RBR pathway. Future research will have to clarify whether this difference is significant.

GCN5 and its cofactor ADA2b are required for stem cell pluripotency and proliferation potential of transit amplifying cells in the PLT pathway

In the shoot, histone acetyltransferase GCN5 has been indirectly linked to expression regulation of homeodomain transcription factor *WUSCHEL (WUS)*, which is required

for shoot stem cell maintenance (Bertrand et al., 2003; Long et al., 2006). We show that GCN5 is also required for root stem cell maintenance through regulation of *PLT* levels (**Chapter 2**). Overexpression of *PLT2* is able to rescue the *gcn5* stem cell defect, which is consistent with the idea that high PLT levels maintain the stem cells (Galinha et al., 2007). The GCN5 associated factor ADA2b similarly promotes *PLT1* and *PLT2* expression. This correlates well with the known function of HATs to quantitatively facilitate transcription (Baker and Grant, 2007; Nagy and Tora, 2007; Shahbazian and Grunstein, 2007). It remains to be resolved whether GCN5 and ADA2b regulate *PLT* levels directly and are present at the *PLT1* and *PLT2* promoter (Figure 1A). Recruitment to the *PLT* promoter could be facilitated by a transcription factor, as occurs in other organisms (Baker and Grant, 2007; Nagy and Tora, 2007).

We further provide evidence that ADA2b influences proliferation of the transit amplifying cells (**Chapter 2**). Possibly, ADA2b mediates the access of PLT proteins to target promoters, analogous to histone demethylase Jmjd1a, which regulates access of the `pluripotent´ stem cell transcription factor Oct4 to a target promoter (Loh et al., 2007). These target genes might encompass cell cycle genes (Sieberer et al., 2003), including *cycB1;1* (**Chapter 2**). High expression of *cycB1;1* is known to enhance shoot and root growth (Doerner et al., 1996; Li et al., 2005a). Therefore, ADA2b might influence the proliferation potential of the transit amplifying cells through regulation of *cycB1;1* expression and redundant family members (Figure 1B). Some cell cycle genes are putative PLT targets (Marijn Luijten and Renze Heidstra; personal communication), consistent with the idea that PLT proteins might have similar targets as ADA2b. The exact molecular mechanism how PLT proteins and ADA2b might regulate cell cycle genes and thereby proliferation of the transit amplifying cells needs further investigation.

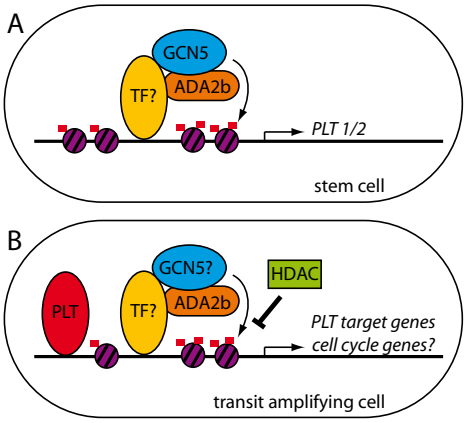
Stem cells are maintained in *ada2b* mutants, despite the reduced *PLT* levels. Determination whether PLT and ADA2b have overlapping target genes in stem cells, should explain this apparent paradox. Previously, it was shown that GCN5 and ADA2b have both overlapping and distinct targets (Vlachonasios et al., 2003) and therefore may not always act in the same complex. Determination of stem cell and transit amplifying cell specific target genes of GCN5 using cell sorting techniques (Birnbaum et al., 2003) may elucidate whether these overlap with ADA2b and PLT targets, and analysis of chromatin complexes on these target genes by ChIP should clarify whether the function of GCN5 and ADA2b depends on their presence in the same complex.

GCN5 and ADA2b could be recruited to PLT target promoters by a transcription factor, similar to their recruitment to cold-regulated genes by the AP2 transcription factor CBF1 (Mao et al., 2006; Stockinger et al., 2001). There are no indications for a direct interaction between GCN5 or ADA2b and the AP2 domain containing PLT proteins (Akie Shimotohno; personal communication), suggesting that GCN5 and ADA2b are recruited independent of PLT proteins (Figure 1B).

In mammals, pluripotent stem cell transcription factors (Oct4 and Sox2) are sufficient to induce the stem cell state (induced pluripotent stem (iPS) cells) and impose the unique chromatin state present in stem cells by regulating chromatin factors (reviewed by Jaenisch and Young, 2008; Niwa, 2007a; Niwa, 2007b). In analogy, the PLT stem cell transcription factors are sufficient to induce ectopic root development including a functional root stem cell niche (Aida et al., 2004; Galinha et al., 2007). Ectopic expression of shoot stem cell transcription factor WUS induces shoot identity in the root (Gallois et al., 2004). Therefore, ectopic expression of key transcription factors PLT and WUS can induce both the stem cell niche and appropriate root or shoot fate, respectively. *plt* mutants support a role for the PLT proteins as root identity factors (Galinha et al., 2007). However, whether WUS is a bona fide organ identity factor is controversial. The WUS homologue WOX5 is specifically expressed in the root organizer or QC and is necessary for root stem cell niche maintenance in the SHR/SCR pathway (Sarkar et al., 2007). Expression of *WUS* in the root stem cell organizer of *wox5* mutants rescues stem cell niche maintenance, but does not affect root fate (Sarkar et al., 2007). These data indicate that WUS and WOX5 act as general stem cell identity factors.

Although Oct4 and Sox2 are sufficient to induce pluripotency, ectopic expression of mammalian oncogenes c-Myc and Klf4 increases the frequency of induced pluripotent stem (iPS) cells (reviewed by Jaenisch and Young, 2008). The transcription factor c-Myc is important for proliferation, stem cell maintenance and is required for the maintenance of active chromatin in progenitor cells by regulating *GCN5* expression directly (Knoepfler et al., 2006). Therefore, GCN5 might contribute to the open and dynamic chromatin state found in stem cells (Meshorer et al., 2006) by providing access for transcription factors to target genes and in this way support the reprogramming process (Jaenisch and Young, 2008). We show that histone deacetylase inhibitor TSA can partially rescue meristem size of *gcn5* and *ada2b*

Figure 1 Model of the GCN5 complex at the promoter of *PLT* genes and PLT target genes



(A-B) GCN5 and ADA2b are probably recruited to the *PLT1, PLT2* promoter (A) and PLT target genes (B), including cell cycle genes, through interaction with an unknown transcription factor. Until now, there are no indications for the recruitment of the GCN5 complex to promoters by PLT proteins, but this will need further investigation. At the promoter, the GCN5 complex acetylates histones (red squares), which opens up the chromatin and promotes transcription. HDACs probably antagonize histone acetylation at PLT target genes (B), and not at *PLT* genes, since *PLT* levels are not elevated upon treatment with TSA.

mutants, which seems to be indirect and independent of *PLT* expression (**Chapter 2**). The *hd1-1* mutant (Tian et al., 2003) does not compensate *gcn5* and *ada2b* meristem size (unpublished data NK), indicating that the responsible HDAC is not HD1 alone and redundant HDACs might be involved. Reduced *PLT* levels in *gcn5* and *ada2b* mutants might be partially compensated by global hyperacetylation or by hyperacetylation of PLT target genes increasing their expression (Figure 1B). Therefore, GCN5 and ADA2b could contribute to active chromatin in root stem cells and their progenitors (transit amplifying cells) as their homologues in mammals may do. Analysis of histone modifications and dynamics of chromatin proteins in stem cells, their daughters (transit amplifying cells) and differentiated cells will elucidate whether *Arabidopsis* stem cells posses a unique chromatin state.

The mammalian pluripotent stem cell transcription factor Oct4 regulates the expression of histone demethylases *Jmjd1a* and *Jmjd2c*, which may play a role in maintaining

open chromatin in stem cells. In turn, Jmjd1a mediates the access of Oct4 to the promoter of self-renewal regulator *Tcl1* (Loh et al., 2007; Niwa, 2007b). Thus, stem cell transcription factors regulate chromatin factors to acquire a special chromatin state in stem cells. In *Arabidopsis*, stem cell associated chromatin factors (HAT GCN5 and remodeller SYD) regulate key stem cell transcription factors (PLT and WUS) (Chapter 2; Kwon et al., 2005), but there is no evidence yet for the opposite regulation, as observed in mammals. Possibly, GCN5 and SYD are necessary to continuously elevate levels of *PLT* and *WUS* to maintain pluripotency in *Arabidopsis* stem cells. In turn, PLT and WUS might regulate *GCN5* and *SYD* expression. There are no indications for a direct regulation of *GCN5* expression by PLT (Marijn Luijten and Renze Heidstra; personal communication). In the future, we will need to determine whether chromatin-associated feedback circuits can be revealed between the PLT proteins and GCN5 in plant stem cells.

Chromatin remodellers and stem cell maintenance

In the shoot, the SNF2 chromatin remodelling factor SPLAYED (SYD) directly regulates *WUS* expression and thereby promotes stem cell maintenance in the shoot (Kwon et al., 2005). Similarly, chromatin remodelling factors are involved in stem cell maintenance in mammals (Lessard et al., 2007) and *Drosophila* (Xi and Xie, 2005). SYD is not involved in root stem cell maintenance (unpublished data NK), suggesting that other SWI/SNF remodellers might be important or that there is redundancy. We find that PICKLE (PKL) is required for root stem cell maintenance, although stem cell loss occurs in a low frequency (unpublished data NK), indicating redundancy with other factors. PKL belongs to the CHD3/Mi-2 type of chromatin remodellers and is known to repress developmental genes (Fukaki et al., 2006; Li et al., 2005b; Ogas et al., 1999; Rider et al., 2004). The role of activating and repressing remodellers in root stem cell maintenance and their relation to stem cell transcription factors and their targets needs further investigation.

The *Arabidopsis* CAF-1 complex, HDACs and RBR are involved in epigenetic stem cell control and differentiation

The *Arabidopsis* chromatin assembly factor-1 (CAF-1) complex, consisting of FASCIATA1 (FAS1), FAS2 and MSI1 is implicated in both shoot and root meristem

maintenance (Kaya et al., 2001). When the CAF-1 complex is defective, the shoot apical meristem is disorganized and *WUS* expression is expanded. We investigated in detail the role of the *Arabidopsis* CAF-1 complex in root development (**Chapter 3**). We show that the CAF-1 complex and RETINOBLASTOMA RELATED (RBR) synergistically repress stem cell proliferation in the root through regulation of stem cell transcription factors (PLT1, WOX5, SCR). Furthermore, the CAF-1 complex and RBR synergistically facilitate differentiation. Previous data show that the CAF-1 complex is involved in proliferation in other plant organs, while it also regulates trichome differentiation (Ramirez-Parra and Gutierrez, 2007a; Schonrock et al., 2006). Furthermore, RBR is involved in differentiation in the *Arabidopsis* shoot and root meristem (Wildwater et al., 2005; Wyrzykowska et al., 2006). In mammals, RB interacts with tissue-specific transcription factors and is known to promote differentiation (reviewed by Skapek et al., 2006). We speculate that the *Arabidopsis* CAF-1 complex and RBR might ensure proper differentiation by regulation of transcription factors involved in differentiation.

We provide evidence that the CAF-1 complex affects stem cell niche maintenance independent of *SCR, SHR* and *PLT* expression and that stem cell loss in *fas* mutants is caused by an accumulation of defects during successive cell divisions (**Chapter 3**). The aberrant *SCR* expression in *fas* mutants as previously reported by Kaya et al. (2001) is not correlated with the early *fas* stem cell niche defect in the primary root. In mammalian ES cells, the CAF-1 complex is essential for correct heterochromatin compaction (Houlard et al., 2006). Similarly, mutation of the *Arabidopsis* CAF-1 complex also causes reduced heterochromatin compaction (Ramirez-Parra and Gutierrez, 2007a; Schonrock et al., 2006). Therefore, the *Arabidopsis* CAF-1 complex might be involved in the maintenance of a special chromatin state in stem cells.

Mutation of the *Arabidopsis* CAF-1 complex causes loss of repressive histone modifications (H3K9me2) at a small subset of genes (Ramirez-Parra and Gutierrez, 2007a; Schonrock et al., 2006). Similarly, our data show that accumulation of epigenetic defects during successive cell divisions and hyperacetylation induced by HDAC inhibitor TSA cause stem cell proliferation (**Chapter 3**). Together, these data suggest that the correct inheritance of repressive histone modifications is important for control of stem cell proliferation and that stem cells might have an active chromatin state. The *hd1-1* mutant (Tian et al., 2003) does not enhance proliferation in the proximal meristem

of *RBR RNAi* seedlings, although it induces additional columella layers (unpublished data NK). Therefore, HD1 is not the main candidate and other redundant HDACs might be involved, which need to be identified in the future.

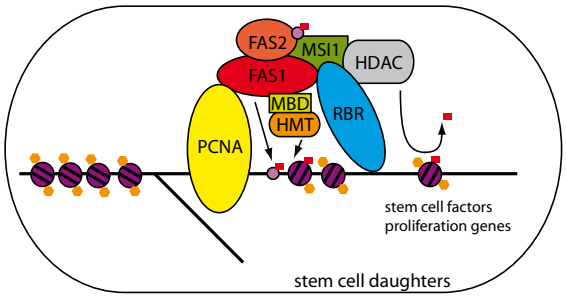
The mammalian CAF-1 complex is present at the replication fork and is involved in nucleosome assembly (Ramirez-Parra and Gutierrez, 2007b; Ridgway and Almouzni, 2000). In addition, the CAF-1 complex is thought to facilitate the correct inheritance of chromatin states at the replication fork through recruitment of several chromatin factors, including HMTs (reviewed by Groth et al., 2007; Wallace and Orr-Weaver, 2005). RB is known to interact with many chromatin factors, including HDACs and MSI proteins (Frolov and Dyson, 2004; Giacinti and Giordano, 2006). In human fibroblasts, RB and the CAF-1 complex are present together at sites of DNA replication in early S-phase (Kennedy et al., 2000). We show that the largest subunit of the *Arabidopsis* CAF-1 complex and RBR interact *in vitro* (**Chapter 3**), opening up the possibility that RBR has two ways to interact with the CAF-1 complex: directly or through MSI1 (Hennig et al., 2003). However, our data show that reduction of MSI1 by 30-50% (Exner et al., 2006) does not affect the phenotype of *fas*, *RBR RNAi* or *fas RBR RNAi* seedlings (Chapter 3), suggesting that MSI1 may not be important. Whether the CAF-1 complex and RBR also interact through MSI1 awaits confirmation.

In conclusion, our data in the root stem cell niche indicate that the *Arabidopsis* CAF-1 complex, RBR and HDACs may be responsible to ensure proper inheritance of repressive epigenetic marks at overlapping targets involved in proliferation and stem cell factors in differentiating daughter cells (Figure 2).

Arabidopsis Polycomb complexes regulate stem cells, transit amplifying cells and cell fate

We provide evidence that the *Arabidopsis* PRC2 Polycomb complex is required for root stem cell niche maintenance (**Chapter 4**). In mammals, the PRC2 complex is involved in H3K27 trimethylation and repression of key differentiation genes in stem cells. Besides the repressive marks, active marks are present (bivalent domains), suggesting that differentiation genes are kept poised for activation upon differentiation (reviewed by Buszczak and Spradling, 2006; Gan et al., 2007; Meshorer and Misteli, 2006). When the PRC2 complex is defective, premature differentiation of stem cells

Figure 2 Model of the CAF-1 complex, HDACs and RB facilitating inheritance of repressive histone modifications



The CAF-1 complex (FAS1, FAS2, MSI1) is recruited to the replication fork through the interaction of FAS1 with replication factor PCNA. The CAF-1 complex loads H3 and H4 onto the newly replicated DNA (light purple small sphere). In addition, it recruits chromatin factors, for example a (DNA) methyl binding domain (MBD)-containing protein and a H3K9 HMT (Reese et al., 2003; Sarraf and Stancheva, 2004), which ensures proper inheritance of H3K9 methylation (orange diamonds). The CAF-1 complex could interact through MSI1 or directly with RBR. In addition, RBR and MSI1 recruit an unknown HDAC, which is probably required to remove histone acetylation from newly synthesized histones (red squares). In this way, the CAF-1 complex, RBR and HDACs ensure correct inheritance of repressive histone modifications at proliferation genes and stem cell factors (like PLT1 and WOX5), which need to be silenced in differentiating stem cell daughters.

takes place. Similarly, we find that mutation of *Arabidopsis* PRC2 Polycomb genes *SWINGER (SWN)* and *CURLY LEAF (CLF)* causes stem cell differentiation, probably due to aberrant expression of key differentiation promoting genes (Figure 3A). Until now, only RBR was shown to promote differentiation in the root (Wildwater et al., 2005). No evidence for the existence of bivalent domains in *Arabidopsis* is available yet, although there are functional Trithorax homologues in *Arabidopsis*, for example ATX1 (Pien and Grossniklaus, 2007). Future analysis should probe the existence of bivalent domains at the promoter of differentiation genes in *Arabidopsis* stem cells and whether *Arabidopsis* Trithorax proteins are involved in stem cell maintenance and counteract PRC2 Polycomb complexes.

SWN and CLF are redundantly required to maintain *SCR* expression (**Chapter 4**), which is in contrast to the general function of Polycomb proteins to repress transcription. In addition, *swn clf* mutants differentiate at the same time as *scr* mutants, indicating

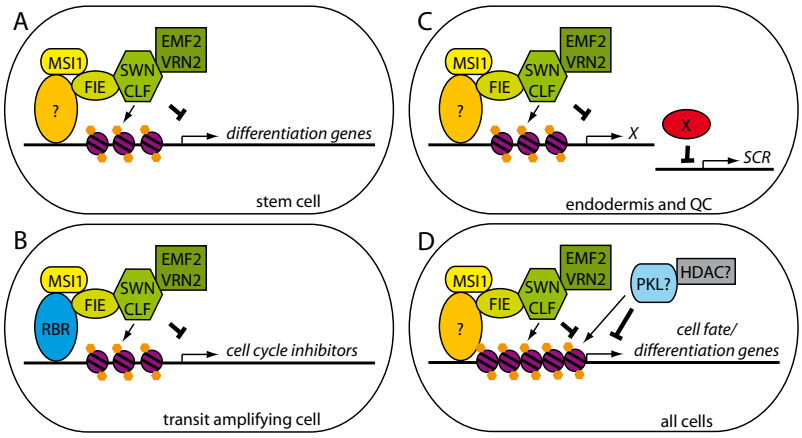
that SWN and CLF act in the SCR pathway. SWN and CLF may be required to repress a negative regulator of *SCR* (Figure 3C), which awaits confirmation in the future.

Our data reveal that RBR is redundantly required with SWN and CLF to stimulate proliferation or prevent differentiation of transit amplifying cells (**Chapter 4**), which needs to be reconciled with previous data that RBR promotes differentiation in the root (Wildwater et al., 2005) and the well-known role of RB in animal systems to restrict cell division and to promote differentiation (Giacinti and Giordano, 2006). SWN, CLF and RBR could synergistically repress specific target genes, involved in proliferation or differentiation of the transit amplifying cells. In mammalian fibroblasts, the PRC1 and PCR2 complexes bind to the *Ink4a*/*Arf* locus, encoding two cell cycle inhibitors in a RB family dependent manner (Bracken et al., 2007; Kotake et al., 2007) and thereby stimulate cell cycle progression (see Chapter 1, Figure 10). Similarly, RBR and *Arabidopsis* PRC2 complexes may together repress cell cycle inhibitors and thereby stimulate proliferation of transit amplifying cells (Figure 3B) and this will be investigated in the future.

Mammalian Polycomb complexes are involved in the maintenance of gene repression, for example at the Hox genes, and in this way shut down alternative genetic programs (reviewed by Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007). Similarly, *Arabidopsis* PRC2 complexes are required to repress transcription and maintain developmental programs (reviewed by Pien and Grossniklaus, 2007; Schubert et al., 2005). For example, root to shoot transformations occur when PRC2 complexes are defective (Chanvivattana et al., 2004; Kinoshita et al., 2001; Schubert et al., 2005). A low frequency of roots in the *pickle* (*pkl*) mutant possess green root tips (Ogas et al., 1997), which resembles the phenotype of PRC2 Polycomb mutants. Animal homologues of PKL are known to repress transcription together with HDACs and there are indications that the same may hold true in *Arabidopsis* (Fukaki et al., 2006). Interestingly, application of the HDAC inhibitor TSA increases the frequency of green roots in PRC2 Polycomb mutants (unpublished data, NK). We postulate that PKL and HDACs might cooperate with PRC2 complexes to repress transcription and maintain developmental programs (Figure 3D).

In mammals, the PRC1 Polycomb complex is recruited to H3K27me3 sites and is thought to further stabilize gene repression (Schuettengruber et al., 2007; Schwartz

Figure 3 Speculative model of PRC2 Polycomb proteins involved in different aspects of development



(A) The PRC2 Polycomb complex is recruited by an unknown factor and ensures repression through H3K27 methylation (orange diamonds) of key differentiation genes in stem cells. (B) The PRC2 Polycomb complex is recruited by RBR and ensures repression of cell cycle inhibitors in transit amplifying cells. (C) The PRC2 Polycomb complex represses transcriptional repressor 'X' and in this way maintains *SCR* expression in endodermis and QC. (D) The PRC2 complex represses key differentiation genes involved in cell fate to maintain the correct developmental program. PKL and associated HDACs could assist by the formation of repressive chromatin.

and Pirrotta, 2007). There is no evidence for a PRC1 complex in *Arabidopsis*. LIKE HETEROCHROMATIN PROTEIN1 (LHP1) is suggested to play a role in the maintenance of gene repression similar to the PRC1 complex (Sung et al., 2006; Turck et al., 2007; Zhang et al., 2007). Despite the severe shoot phenotype of *lhp1* mutants, no evidence for a role of LHP1 in root stem cell maintenance can be found (unpublished data NK).

RB may act as a platform coordinating stem cell maintenance, proliferation and differentiation together with chromatin factors

RB interacts with many chromatin factors, which are thought to mediate its transcriptional regulation (Frolov and Dyson, 2004; Giacinti and Giordano, 2006). In general, RB is known to prevent cell cycle progression through repression of E2F target genes in animals (Giacinti and Giordano, 2006) and the same is found in plants

(De Veylder et al., 2007; Desvoyes et al., 2006; Inze and Veylder, 2006). However, besides cell cycle regulation, RB promotes differentiation and interacts with tissue-specific transcription factors in animals (Korenjak and Brehm, 2005; Skapek et al., 2006). In *Arabidopsis*, local overexpression of *RBR* in the shoot and root meristem induces differentiation (Wildwater et al., 2005; Wyrzykowska et al., 2006). Reduction of *RBR* in the root delays differentiation, resulting in retention of cell division capacity in stem cell daughters (Wildwater et al., 2005). In line with these data, we find that the *Arabidopsis* CAF-1 complex and RBR synergistically repress stem cell proliferation and promote differentiation (**Chapter 3**). In addition, we provide evidence for a novel function: RBR and *Arabidopsis* PRC2 Polycomb complexes may redundantly promote proliferation of the transit amplifying cells (**Chapter 4**). We postulate that the diverse functions of RBR (i.e. promotion and repression of proliferation and induction of differentiation) are important in different regions of the root and might be mediated by post-translational modifications of RBR and association with different chromatin complexes.

The histone chaperone MSI1 is found to interact in plants and animals with the CAF-1 complex, HDACs, PRC2 complex and RB (reviewed by Hennig et al., 2005). RB directly binds to HDACs in humans and maize (Frolov and Dyson, 2004; Giacinti and Giordano, 2006; Rossi et al., 2003). In mammalian fibroblasts, the PRC1 and PCR2 complex bind to the *Ink4a*/*Arf* locus in an RB family dependent manner (Bracken et al., 2007; Kotake et al., 2007). In *Arabidopsis*, RBR interacts with the PRC2 Polycomb protein FIE (Mosquna et al., 2004). RBR interacts *in vitro* with the largest subunit of the *Arabidopsis* CAF-1 complex (**Chapter 3**). In addition, mutation of PRC2 Polycomb complexes (**Chapter 5**), and acute reduction of *RBR* (Wildwater et al., 2005) and the middle subunit of the CAF-1 complex (Chapter 3) cause root cap bending. In general, the available data suggest that RBR, the CAF-1 complex, HDACs and PRC2 Polycomb complexes may act together in the root. It will have to be established which chromatin factors are present in the same complexes *in vivo* and whether they have overlapping functions and targets. Determination, verification and comparison of RBR and Polycomb stem cell and transit amplifying cell targets by micro-array and ChIP analysis as well as examining the joint presence of these factors, the CAF-1 complex and HDACs at target promoters by ChIP and IP will be the next step in understanding epigenetic control of stem cells and transit amplifying cells in roots.

In conclusion, regardless of the precise mechanisms involved, our finding that root stem cells are very sensitive to changes in chromatin factors suggests that *Arabidopsis* stem cells might possess a specialized chromatin state that requires fine-tuning by epigenetic mechanisms. Our data open up the intriguing possibility that similar classes of chromatin factors are involved in stem cell niche maintenance in both plants and animals. This suggests that chromatin factors have been recruited as essential players for stem cell maintenance, even though multicellularity arose independently in both kingdoms. In mammals, pluripotent stem cell transcription factors probably regulate chromatin factors to obtain a special chromatin state in ES cells. Similarly, in *Arabidopsis*, stem cell transcription factors are sufficient to induce root or shoot stem cells and it is an interesting possibility that they regulate chromatin factors, whereas it is already evident that chromatin factors regulate stem cell transcription factors. If true, it remains to be seen whether feedback circuits between chromatin factors and transcription factors have been invented twice during evolution of multicellular plants and animals or whether chromatin factors were already the hallmark of proliferative stages of a common unicellular ancestor.

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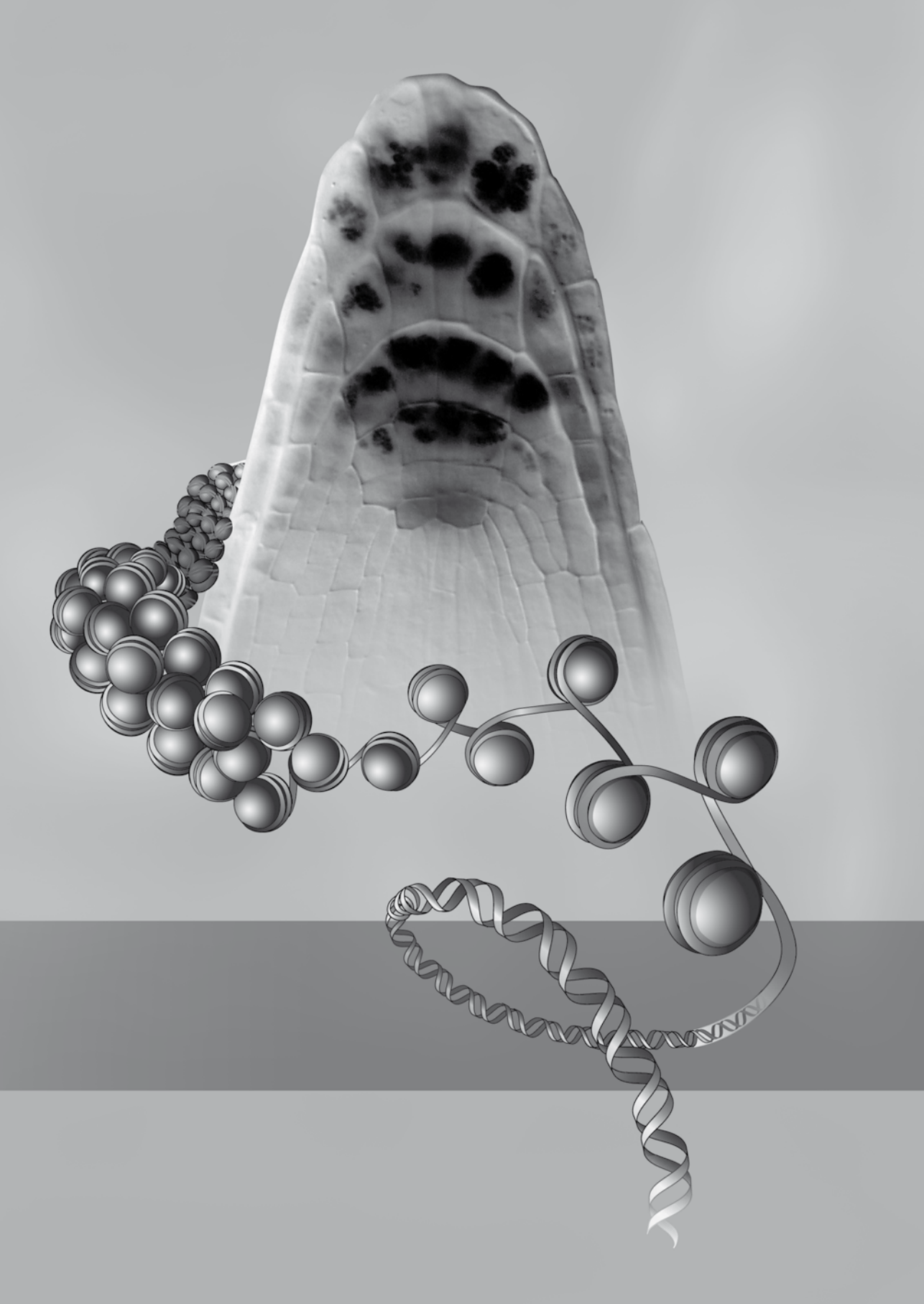

Summary / Samenvatting

Summary

Stem cells replenish the cells present in an organism throughout its lifetime and sustain growth. They have unique characteristics: the capability to self-renew and the potential to differentiate into several cell types. Recently, it has become clear that chromatin factors support these unique features in mammalian stem cells (**Chapter 1**). The role of chromatin factors in plant stem cell control is just starting to be revealed. In this thesis, we describe investigations on the roles of chromatin factors in *Arabidopsis* root stem cell maintenance. We performed a genome-wide survey of chromatin factor to identify which classes of chromatin factors could be genetically linked to stem cell action. In addition, we investigated the relationship of these chromatin factors with existing stem cell regulatory pathways. We find that several classes of chromatin factors (histone acetyltransferase GCN5, the CAF-1 complex, histone deacetylases (HDACs), PRC2 Polycomb proteins) regulate root stem cell niche maintenance.

We show that GCN5 affects root stem cell niche maintenance mainly through expression regulation of stem cell transcription factor *PLETHORA (PLT)* (**Chapter 2**). Overexpression of *PLT2* is able to rescue *gcn5* mutants. The GCN5 associated factor ADA2b also regulates *PLT* expression and acts in the PLT pathway, but is not involved in stem cell niche maintenance. Instead, we provide evidence that ADA2b mediates meristem size and proliferation of the transit amplifying cells directly.

Furthermore, we demonstrate that the chromatin assembly factor 1 (CAF-1) complex and RETINOBLASTOMA RELATED (RBR) repress synergistically root stem cell proliferation through regulation of stem cell niche transcription factors and promote correct differentiation (**Chapter 3**). Genetic interactions position the CAF-1 complex in the SHORTROOT (SHR)/ SCARECROW (SCR) pathway. We provide evidence that the CAF-1 complex and RBR may ensure propagation of repressive epigenetic marks at overlapping target genes together with HDACs and in this way play an important role in epigenetic stem cell control.

In addition, we show that PRC2 Polycomb proteins SWINGER (SWN) and CURLY LEAF (CLF) are redundantly required to maintain the stem cells and expression of the root stem cell transcription factor *SCR* (**Chapter 4**). Our data reveal a novel function for RBR: it is redundantly required with SWN and CLF to stimulate proliferation or prevent differentiation of the transit amplifying cells.

Finally, the results described in this thesis are discussed in light of recent insights into the specialized chromatin state in stem cells (**Chapter 5**).

In conclusion, the research performed in this thesis reveals that several classes of chromatin factors are required for root stem cell maintenance and play a role in both parallel pathways known to define the root stem cells. In mammals, pluripotent stem cell transcription factors probably regulate chromatin factors to obtain a special chromatin state in ES cells. Similarly, in *Arabidopsis*, stem cell transcription factors are sufficient to induce root or shoot stem cells and it is an interesting possibility that they regulate chromatin factors, whereas it is already evident that chromatin factors regulate stem cell transcription factors.

Samenvatting

Chromatine is het complex van eiwitten (histonen) waar omheen DNA is gewikkeld (zie voorkant van het proefschrift) en andere geassocieerde eiwitten. Aan de histonen kunnen verschillende moleculen (histon modificaties) gehangen worden door middel van een verscheidenheid aan chromatine factoren (enzymen) (Hoofdstuk 1, Figuur 1), bijvoorbeeld histone acetyltransferases die acetylgroepen aan de histonen hangen. Dit beïnvloedt de compactheid van het DNA (zie voorkant van het proefschrift) en de productie (expressie) van eiwitten die onder andere betrokken zijn bij groei en identiteit (bv. wortel of blad, hand of voet) (**Hoofdstuk 1**).

Stamcellen zijn van belang voor de groei van een organisme en het behoud van de weefsels. Ze hebben unieke eigenschappen: het vermogen om zichzelf in stand te houden en de capaciteit tot het vormen van verschillende celtypen (bv. bloemblaadjes of meeldraden). Stamcellen zijn niet alleen in meercellige dieren en mensen te vinden, maar ook in planten. Meercellige dieren/mensen en planten stammen af van ééncellige voorouders. Dit betekent dat stamcellen waarschijnlijk twee keer zijn uitgevonden: in dieren/mensen en in planten. De stamcellen van *Arabidopsis thaliana* (zandraket) bevinden zich op twee plekken in zogenoemde meristemen: in de scheut en in de wortel. De scheutstamcellen vormen alle bovengrondse delen en de wortelstamcellen vormen alle ondergrondse delen, het wortelstelsel (**Hoofdstuk 1**).

Voor het onderzoek dat beschreven is in dit proefschrift is de wortel van *Arabidopsis* gebruikt, vanwege de makkelijk te identificeren stamcellen (Hoofdstuk 1, Figuur 15). Eerder onderzoek laat zien dat een aantal plantspecifieke transcriptiefactoren (eiwitten), zoals PLETHORA (PLT), SHORTROOT (SHR) en SCARECROW (SCR), belangrijk zijn voor het definiëren van de wortelstamcellen (Hoofdstuk 1, Figuur 15). Daarnaast reguleert het eiwit RETINOBLASTOMA RELATED (RBR) de hoeveelheid stamcellen in de wortel. RETINOBLASTOMA (RB) is betrokken bij de regulatie van celdelingen (celcyclus), ook in planten, en het is een tumor suppressor in dieren en mensen. Als stamcellen delen dan blijft één cel in contact met de “organizer” of quiescent centre (QC) waardoor deze de stamcel identiteit behoudt en de andere cel (dochtercel) differentieert (transformeert) in een specifiek celtype (bv. vaatweefsel of wortelmutsje (root cap)). Sommige dochtercellen (in het proximale meristeem) kunnen nog een beperkt aantal keer delen voordat ze differentiëren en worden “transit amplifying cells” genoemd (**Hoofdstuk 1**).

Recentelijk is in dieren aangetoond dat chromatine factoren belangrijk zijn voor het behoud van de stamcelstatus (**Hoofdstuk 1**). In dit proefschrift heb ik onderzocht welke klassen chromatine factoren betrokken zijn bij stamcelbehoud in de wortel van *Arabidopsis* door te kijken wat er gebeurd met de stamcellen als chromatine factoren uitgeschakeld (gemuteerd) worden. Deze planten zijn dan mutant voor een chromatine factor. Daarna heb ik bestudeerd wat de relatie is tussen de gevonden chromatine factoren en de transcriptiefactoren (PLT, SHR en SCR) en RBR waarvan al bekend is dat ze wortelstamcellen reguleren.

In **hoofdstuk 2** laat ik zien dat de chromatine factor GCN5 nodig is om de wortelstamcellen te behouden. GCN5 is een histon acetyltransferase, die acetylgroepen aan de histonen hangt en daardoor het DNA en de chromatine "open" maakt. Als deze chromatine factor uitgeschakeld is (in de *gcn5* mutant) verdwijnen de stamcellen. Bovendien is specifiek de hoeveelheid (expressie) van de stamcel transcriptiefactor PLT verlaagd, maar niet van SHR en SCR. Als ik de hoeveelheid PLT weer verhoog (overexpressie) in de wortel van de *gcn5* mutant, dan blijven de stamcellen behouden. Dit betekent dat de chromatine factor GCN5 de hoeveelheid (expressie) van PLT reguleert en daardoor stamcellen behoudt. Ook heb ik de functie van de chromatine factor ADA2b bekeken, waarvan bekend is dat deze geassocieerd is met GCN5. Als ADA2b uitgeschakeld is (in de *ada2b* mutant), is de hoeveelheid (expressie) van PLT specifiek verlaagd en is het meristeem kleiner, maar de stamcellen worden behouden. Deze resultaten zijn verrassend. Verder onderzoek aan stamcellen is nodig om dit te verklaren. Normaal leidt een verhoging van de hoeveelheid (overexpressie) van PLT tot een vergroot meristeem (omdat er meer delingen plaatsvinden in de transit amplifying cells), maar dit gebeurt niet in de *ada2b* mutant. Dit betekent dat ADA2b het "uitlezen" van de hoeveelheid PLT reguleert en de delingen in de transit amplifying cells. De huidige hypothese is dat ADA2b de PLT "downstream targets" reguleert die de delingen in de transit amplifying cells beïnvloeden (Hoofdstuk 5, Figuur 1). Ook hier is verder onderzoek nodig in de toekomst om het exacte mechanisme vast te stellen.

In **hoofdstuk 3** beschrijf ik hoe het chromatin assembly factor 1 (CAF-1) complex stamcellen controleert. Het CAF-1 complex is waarschijnlijk betrokken bij het opbouwen van chromatine nadat het DNA verdubbeld is tijdens celdeling, zodat dit correct overgeërfd wordt (Hoofdstuk 5, Figuur 2). Dat doet het CAF-1 complex in

dieren waarschijnlijk door het rekruteren van andere chromatine factoren. Als het CAF-1 complex uitgeschakeld (gemuteerd) is (in de *fas* mutanten) en als de hoeveelheid RBR verlaagd wordt, dan gaan de stamcellen overmatig delen en is de hoeveelheid (expressie) van stamcel transcriptiefactoren (bv. PLT, SCR) ontregeld. Iets soortgelijks vindt ook plaats als tegelijkertijd de hoeveelheid RBR verlaagd wordt en als histone deacetylases (HDACS, chromatine factoren die acetylgroepen verwijderen) geremd worden. Mijn resultaten laten zien dat waarschijnlijk de overerving van histon modificaties verkeerd gaat die normaal zorgen dat stamcelfactoren en delingsfactoren "uit" zijn in stamceldochters. Het exacte mechanisme hoe het CAF-1 complex, RBR en HDACS stamcellen reguleren zal worden ontrafeld in de toekomst.

In **hoofdstuk 4** toon ik aan dat Polycomb chromatine factoren (SWINGER and CURLY LEAF) essentieel zijn voor stamcelbehoud in de wortel. Eerder onderzoek laat zien dat Polycomb eiwitten nodig zijn om de identiteit (cell fate) te behouden van weefsels en dat ze belangrijk zijn voor stamcelbehoud in dieren. De hoeveelheid (expressie) van stamcel transcriptiefactor SCR is verlaagd in een aantal wortels als het Polycomb complex defect is en dit suggereert dat daardoor stamcelverlies plaatsvindt. Bovendien laten mijn resultaten zien dat RBR en het Polycomb complex samen de transit amplifying cells beïnvloeden, en dit onthult een nieuwe functie van RBR. De exacte rol van het Polycomb complex in stamcellen en transit amplifying cells zal in de toekomst verder onderzocht moeten worden.

In **hoofdstuk 5** bediscussieer ik de resultaten beschreven in hoofstuk 2, 3 en 4 in relatie tot de meest recente kennis op het gebied van chromatine en stamcellen.

In conclusie, mijn onderzoek toont aan dat verschillende soorten chromatine factoren betrokken zijn bij stamcel behoud in de *Arabidopsis* wortel. Stamcellen zijn erg sensitief voor defecten in chromatine factoren en dit suggereert dat stamcellen in de wortel een speciale chromatine status hebben, zoals recente onderzoeken in dieren ook aangeven. De chromatine factoren die betrokken zijn bij stamcelbehoud die ik gevonden heb zijn vergelijkbaar met de chromatine factoren in dieren die essentieel zijn voor stamcellen. In dieren, expressie van transcriptiefactoren is voldoende om de stamcelstatus te induceren en hetzelfde geldt voor planten. In dieren, transcriptie-factoren reguleren chromatine factoren om een speciale stamcelstatus te verkrijgen. Mogelijk vindt hetzelfde plaats in planten. Ik heb gevonden dat chromatine factoren

de expressie van stamcel transcriptiefactoren reguleren en verder onderzoek zal moeten uitwijzen of dat ook in dieren het geval is. Samengevat opent mijn onderzoek de weg voor een verdere exploratie van de rol van chromatine factoren in stamcellen.

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Noor

Curriculum Vitae

Noortje Georgina Kornet (kortweg Noor) werd geboren op 13 november 1978 te Wageningen. Na het behalen van het VWO diploma in 1997 aan het Pantarijn te Wageningen (ook wel bekend als “het Wagenings”), begon zij aan de studie Biologie aan de Wageningen Universiteit. Tijdens de specialisatie in moleculaire en cellulaire biologie heeft ze verschillende stages gelopen. De eerste stage was bij de vakgroep Moleculaire Biologie, waar ze het ‘movement protein’ van het Cowpea Mosaic Virus, een plantenvirus, onderzocht. De tweede stage was bij Molecular Cell Biology aan de Johann Wolfgang Goethe-University, Frankfurt am Main, Duitsland. Daar heeft ze de rol van hittestress factoren bestudeerd in tabak- en tomatenplanten. De laatste stage vond plaats in het Laboratorium voor Microbiologie. Zij werkte aan een archaea-specifieke transcriptie regulator uit *Sulfolobus solfataricus* betrokken bij koper homeostase. Nadat zij in 2002 cum laude afstudeerde, begon zij op 1 april 2002 als assistent in opleiding bij de vakgroep Moleculaire Genetica aan de Universiteit Utrecht onder begeleiding van Prof.dr. Scheres. Hier bestudeerde zij de rol van chromatinefactoren in stamcelbehoud in de *Arabidopsis* wortel, waarvan de resultaten beschreven staan in dit proefschrift. Vanaf 1 augustus 2008 zal zij werkzaam zijn in de groep van Prof.dr. Laux, Molecular Genetics and Biotechnology of Plants, University of Freiburg, Duitsland.

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